MECHANISMS OF HEAVY METAL UPTAKE IN A MIXED MICROBIAL SYSTEM

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ABSTRACT

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The potential use of mixed microbial systems for the removal of heavy metals (Cr, Pb, and Se) from contaminated water was investigated. The study included the development of metal-tolerant mixed microbial strains for increased metal recovery and the investigation of the uptake mechanisms. The results showed that the growth dynamics during mat formation resulted in a typical two microbial phase pattern: (1) a mixed bacterial bloom, during which the metal ions were mobilized for uptake, and (2) a cyanobacterial phase, a period marked by the stabilization of the existing metal species in the floating mixed microbial mat. Investigations on the uptake mechanisms suggested that reduction by microbial sulfate reactions, precipitation of metal ions, adsorption at membrane sites, reduction by change of oxidation states, and chelation played major roles in describing the strategies of bacterial response to the heavy metals investigated.

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CHAPTER I

INTRODUCTION

Contamination of surface water by conglomerate mixtures of heavy metals is a significant problem in aquatic environments near mining and metal processing operations. The development of mixed microbial systems for metal uptake will be a significant step in decontaminating water and conserving metal resources by reclamation and recycling. In addition, when analyzing the potential system cost, it is important to consider that cyanobacteria (one of the primary microbes in the system) fix both nitrogen and carbon, and therefore will increase the nutritional pool of the ecosystem.

The need of using microorganisms for the removal of heavy metals from aquatic environments has long been an attractive idea, not only for water and wastewater purification, but for the recovery and recycling of valuable or economically important metals. The feasibility of such a biological approach in metal recovery, as an exploitable process is indisputable because of the intrinsic capacity of microorganisms to bioconcentrate metal ions from solutions, (Tuovinen and Kelly, 1974; Kelly et al., 1979).

Several mechanisms of microbial bioconcentration and recovery of heavy metals have been investigated (Hart and Scaife, 1977). The specific degree of toxicity of each

metal, however, determined the use of such microbes in metal recovery applications. Hong-Kang and Wood (1984) have suggested that microorganisms may adopt a number of strategies to maintain a low intracellular concentration of the metals, these include:

1. The binding of metal ions to cell surfaces.
2. The biosynthesis of intracellular polymers which serve as traps for the metal ions.
3. The biomethylation and transport of metal ions through cell membranes by a diffusion-controlled process.
4. Volatilization.
5. The extracellular complexing and subsequent accumulation of metal ions.
6. The precipitation of insoluble metal complexes (such as PbS) at cell surfaces.

Noris and Kelly (1985) have demonstrated that the mechanism of metal uptake takes place in two distinct stages. The first stage has been described as a passive adsorption of ions and it is likely that a number of different functional groups are involved, (Crist et al., 1981; Beveridge and Koval, 1981). The second stage of uptake is slower, and may involve active transport mechanisms, which require cellular energy (Kaback, 1987).

Applying the above principles, Shumate et al. (1980) used Saccharomyces cerevisiae and Pseudomonas aeroginosa for the bioconcentration of uranyl ions. Each microorganism was capable of accumulating 100 to 150 mg

uranyl ions/g dry weight. Similarly, (Tsezos and Volesky, 1981, 1982) used industrial by-products of Rhizopus arrhizus biomass as a biosorbant of thorium. The results of which were inconsistent after repeated trials. Norberg and Person (1984) have also used the biomass of Zooloea ramigera for the uptake of cadmium, uranium, and copper.

Some of these previous studies have their good potentials, but are highly limited due to the variables that are often encountered in wastewater. Wastewaters often contain a variety of toxic substances, including a wide range of heavy metals, which cause fluctuating pH conditions. It is therefore, difficult to maintain microbial growth over a long period of time.

Understanding these limitations, and also, the possible mechanisms of metal uptake by microorganisms in relationship to their growth, this present study resorted to the development of simulated pond systems. In this system, metal-adapted mixed microbes cultured on silaged grass, generated a microbial mat which primarily served as a "filter" for the accumulation of metal ions. The mixed microbial system described here also employed the advantages of utilizing a complete and integrated ecosystem. The system is unique in the sense that the entire environment becomes functional. This is significant since, as a result, the metal uptake is not dependent on the specific role of any set of microbes, rather both the chemical environment and the mixed

microbes work in concert for an efficient removal of toxic metal ions. Therefore, the objectives of this study were: (1) Development of metal-tolerant bacterial strains. The development of such microbes directly affected the maintenance of the mixed microbial culture, and consequently, increased the stability of the biomass and uptake dynamics of the metals in the system, (2) Physicochemical analyses of the mixed microbial mat and (3) Investigation of some of the mechanisms of metal mobilization by bacteria. This section was addressed under (i) metal chelation, (ii) metal adsorption, (iii) metal absorption, and (iv) metal precipitation.

CHAPTER II

REVIEW OF LITERATURE

In recent years, wastewater treatment processes have assumed an important status in the need for a more controlled quality environment. Accepted terminology for all those processes which handle pollutants has gradually changed from "wastewater disposal" through "wastewater treatment" to "wastewater reclamation" thus reflecting a change in attitude towards wastewater as a vital natural resource (Brewin and Hellawell, 1980).

The heavy metals are an important group among the pollutants present in wastewater, and their effects on natural microbial communities have attracted increased attention. They (heavy metals) are added to the environment from a variety of sources, including municipal, industrial and agricultural wastes (Kinkle et al., 1987). The magnitude of the problem concerns not only direct toxicity to humans by water contamination or through bioconcentration in the food chain, but also relates to changes in the natural environmental processes. Heavy metals that interact at the microbial level may interfere with fundamental processes essential to life of any aquatic system. This is because phytoplankton form the base of the food chain, and thus have the potential of transferring heavy metals through tropic levels (Hart and Scaife, 1977). The extent to

which phytoplankton may transfer metals depends in part on their ability to accumulate and to a larger degree, tolerate high concentrations of the metal before they become affected.

Microbial response to heavy metals may vary with the species and may be complicated by the nature of both the absorbent and the metal species in aqueous solutions, especially in wastewater. Microbial cell walls contain many potential sites for the binding of ions (Crist et al., 1981), and it is unlikely that any one type of molecule or functional group is responsible for the absorption of the metal ions. Microbial cell surfaces are usually charged. The anionic ligands responsible for cation adsorption include phosphoryl, carboxyl, sulfhydryl and hydroxyl groups of membrane proteins and lipids and of cell wall structural components such as bactererial peptidoglycan and associated polymers (Beveridge and Koval, 1981; Doyle et al., 1980; and Kelly et al., 1979).

It is clear therefore, that the chemical mechanisms one would expect to operate in the speciation or precipitation of the various metals are important in describing the chemical environment of the mixed microbial system. The results of many studies have disclosed a tremendous variation on what type of metal species will prevail in solution (Hahn and Welcher, 1980). This variation may be expected to be more complex in a mixed microbial system, more so within the mat.

Some of these variations include particle size, nucleation, pH, concentrations of the inorganic or organic salts present in the culture media, and oxidation-reduction states (Hahn and Welcher, 1980). Therefore, the speciation or precipitation of any metal will depend to a large extent upon the chemistry of the metals, the surrounding chemical environment, and activities of the microbial flora in the system. In general, it is believed that the higher valent cations form the most insoluble hydroxides and therefore will precipitate at lower pH values (Hahn and Welcher, 1984).

Inorganic lead speciation is dominated by the (+2) oxidation state rather than the tetravalent (+4) oxidation state (Hahn and Welcher, 1980). Depending on the amounts of salts present in the medium, (Hahn and Welcher, 1984) have shown that Pb can form the following precipitates: $PbCl_2$, $Pb(OH)_2$, $Pb(PO_4)_2$, and PbS. Most inorganic salts are sparingly soluble (PbF_2 and $PbCl_2$) or virtually insoluble lead sulfate in water. Exceptions are lead acetate and lead nitrate.

On the other hand, the organolead compounds are different in their speciation. The difference, primarily is due to the lead-carbon (Pb-C) bond energy of 130 Kcal/mole (Shaw and Alfred, 1970) which is twice the lead- hydrogen (Pb-H) value that exists in inorganic lead compounds. Consequently, as suggested by Demeester and Hudgon (1976) the organic chemistry of lead is dictated primarily by the tetravalent (+4) oxidation state.

Therefore, in a mixed microbial system, comprising of several different microenvironments, one may expect the prevalence of these major organolead compounds: tetramethyl-lead, tetraethyl-lead, tetraphenyl-lead hexamethyl-lead and hexaethyl-lead (Shapiro and Frey, 1968).

The complex formation and chelation properties of the organolead derivatives are also of importance in lead speciation. Due to the small differences in the electronegativities of lead and carbon (Dyrssen, 1972), mixed microbial systems may contain covalent bonding (donor-acceptor type, in which both electrons in the bonding orbital originate from the carbon atom) between lead and carbon compounds. In this case, the metal occupies a central position in the complex, as exemplified by the lead atom in tetramethyl-lead, which is surrounded by four methyl groups.

Basolo and Pearson (1986) had earlier demonstrated that lead can form strong bonds to sulfur in the cysteine residues of proteins and enzymes; they also demonstrated the ability of lead to bind strongly to imidazole groups of histidine residues and with the carboxyl groups of glutamic and aspartic acids. In this context, one may expect lead to react with living organisms by binding to peptide residues in proteins, thus preventing the proteins from carrying out their functions by changing their tertiary structures or by blocking the active site of an enzyme. Therefore, depending on the stoichiometry

and structure of the chelate and on the pH of the environment (Wong, 1973; Carty, 1976) chelates can play a useful role of competing with the peptides for the metal by forming stable chelate complexes. Such complexes can then be transported from the protein and excreted extracellularly.

Results of several studies (Rosenfeld, 1984) suggest that the positive oxidation states of selenium are +4 and +6, and only a few unstable compounds are in +2 states. The binding in these states is primarily covalent. In the +4 state, selenium shows both reducing and oxidizing properties, while in the +6 state, selenium has only oxidizing properties. In selenides, selenium assumes the oxidation state of -2 (Rosenfeld, 1984). Biotransformation of selenium in aquatic environments, including soil, sediment and sewage have been reviewed by Doran (1982). Results of this and other reviews (Chau et al., 1976; Reamer, 1980) suggest that the addition of selenate or selenite to lakes or sediments, results in the emission of very volatile methylated selenium species, including the product of insoluble selenium metal. Also recently, Burton et al.(1987) identified the deposition of elemental selenium at the Kesterson reservoir in California which was attributed to the presence of a high incidence of selenite-resistant bacteria in the area.

Unlike lead and selenium, the speciation of chromium is different. Chromium is a transition metal that shows

several oxidation numbers; the principal ones are +2, +3, and +6 (Hahns and Nostrand, 1980). There are two anions in which the oxidation number of chromium is +6. These are the chromate and the dichromate ions. Both of these are present in aqueous solutions. If the solution is basic, the chromate ions predominate, whereas at low pH values, the dichromate prevails (Hahns and Nostrand, 1980). Chromous ion (Cr^{2+}) can also form at low pH but can be oxidized immediately to deep green chromic ions in the presence of air. Christensen and Delwiche (1981) had successfully shown that the reduction of hexavalent chromium ions to their trivalent states are necessary procedures during chromium treatment. In this context, the overall speciation of chromium becomes very significant in its uptake.

It is apparent from the above literature review that metal speciation behavior is important in the application of biological systems in metal uptake and in the understanding of the mechanism of the process. Mixed microbial systems represent a complex and a unique ecosystem, both in the chemical content of the various components (soil, water column, and the microbial flora). The complexity of the entire system is further varied due to the existence of microenvironments. The presence of such microenvironments may constitute a normal biotic phenomena, since organisms can only prevail in niches that support their existence. In this context, one may expect that the microenvironments present in the soil or

sediment should be chemically different from those of the water column and the floating microbial mat (EPA, 1977). For example, lead has been shown to be precipitated at anaerobic regions found at the microbial mat (Bender et al., 1988).

Therefore, the survival and the efficiency of any microbe in these microvironments may depend to a large degree to the level of toxicity of the various metals and to the corresponding resistance expressed by the microbes. Wang and Wood (1983) have indicated that the biochemical basis for resistance to metal toxicity is complicated by the great variety of reactions at the molecular and cellular levels, even in closely related organisms and tissues. Williams (1983) has pointed out that heavy metal interactions in biological systems can be divided into three classes: ions in fast exchange with biological ligands, ions in intermediary exchange with biological ligands and ions in slow exchange with biological ligands. Metal ions such as Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} are grouped as the elements in the fast exchange, while, Fe^{2+} and Mn^{2+} are in the intermediary category. The slow exchangers were identified as Zn^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+}. Based on this classification, (Williams, 1983) also suggested that metal-to-metal interactions and covalency predominate in the exchange metals, and this as pointed out by the same author, forms the basis for metal species stability in aquatic systems.

A comparative study of metal ion resistance between prokaryotes and eukaryotes, has revealed the importance of membranes to metal ion resistance (Silver et al., 1981). The external cell membranes of prokaryotes are known to carefully select those ions at the fast exchange while the eukaryotes are much more resistant to ions at the slow exchange. However, over the years, following the rapid industrialization, and the subsequent huge changes in the distribution and solubilization of metal ions, microorganisms have acquired enhanced ability to resist high metal concentrations. Some of these strategies of microbial resistance to heavy metals have been identified (Wong and Wood, 1984).

One such strategy has been proven by Barkay (1987) while studying the mechanism of adaptation to Hg^{2+} in four aquatic habitats. Among the several strategies, microbial volatilization of Hg^{++} was identified as a major adaptive mechanism for Hg^{++} toxicity. On the other hand, Gauthier et al. (1985) have demonstrated the presence of plasmids among the thirty-one strains shown to be resistant to mercury. Similar correlations to plasmid-mediated resistance to various metals have been documented (Nakahara et al., 1977; Laube et al., 1980; Capone et al., 1983; Calomiris et al., 1984; Zehr et al., 1987).

In general, microbial resistance to heavy metals usually results in the transformation of the metals

(Summers and Silver, 1978). In their review, Summers and Silver (1978) have reported that biotransformation can be divided into two broad categories: (a) redox conversions of inorganic forms, and (b) conversions from inorganic to organic form and vice versa. Metals such as arsenate, iron, mercury, and selenite have been implicated in these categories of transformations. Of interest is the role of bacteria in methylation of metals because of the enhanced toxicity of the methylated forms of many metals (Jernelov and Martin, 1975). Due to this concern, several studies have been documented in this area, primarily at the ecological level (Chopra, 1975; Huey et al., 1978; Inverson and Brinkman, 1978). Despite the progress made in this area, research into the biochemical mechanisms and genetics of transformations has progressed slowly in some cases and more rapidly in others.

Among the metals, mercury (Hg) stands as the most extensively studied. The chemistry of mercury and its organomercurial detoxification by bacteria have been widely reported by several workers. Data from these early investigations suggest the existence of three basic mechanisms of resistance: synthesis of mercury-binding thiols (Ross, 1973); permeability barriers which limit access of the mercury into the cell (Brierley, 1978); and the elimination of the toxic mercurial ion from the growth medium (Tezuka and Tonumura, 1978) In every case, Hg^{++} was converted to a volatile form that subsequently was identified as Hg^{o} (Furukawa et al., 1971; Summers and

Silvers, 1972). The molecular basis for this mercurial transformation has been implicated to mercury (ii) reductase. This enzyme [mercury (ii) reductase] has been purified from the soil isolate Pseudomonas K62 and from two plasmid-containing Escherichia coli strains (Farukawam et al., 1971; Schottel, 1978). In all cases tested, the mercuric reductase was found in the cytosol (Summers and Sugarman, 1974).

Lead (one of the metals investigated in this present study) like mercury is highly toxic and forms one of the metals promiscuously discharged into the environment. However, relatively few data exist on the biochemical pathways involved in its mechanism of biotransformation. Wong et al. (1975) first gave the evidence of methylation of lead in the environment. However, the pathways for the biological conversion were not well understood. It was apparent that the conversion of inorganic lead to organic lead is a difficult process and probably requires specific physical, chemical, as well as biological conditions. On the other hand, the biotransformation of trimethyl-lead to tetramethyl-lead seem to proceed readily. This fact was supported by Wong et al. (1975) while using pure bacterial isolates from Lake Ontario. The report found that species of Pseudomonas, Alcaligenes, Acinetobacter, Flavobacterium, and Aeromonas were able to transform trimethyl-lead acetate to tetramethyl-lead. None of the isolates was able to produce tetramethyllead from inorganic lead.

Following the results of Wong et al. (1975), Jarvie et al. (1975) demonstrated the possible chemical alkylation of lead by anaerobic micro-organisms. The significance of these data was the suggestion that the main reaction producing tetraalkyl-lead from trialkyl-lead in anaerobic system is the conversion of the trialkyl-lead salt to the sulfide, and decomposition of the trialkyl-lead sulfide to form the tetra-alkyl-lead. This premise was supported by Schmidt and Huber (1976). Similar studies by Reamer and Zoller (1980) also suggested the biomethylation of selenium compounds by micro-organisms found in sewage sludge and soil. Selenium is of interest as a potential environmental toxicant because of the small margin between nutritional levels and human toxicity (Shroder and Frost, 1970). Molecular mechanisms of selenium reduction suggest that an auxotroph of Psedomonas aeroginosa contains a protein which has a site capable of substituting selenium for sulfur in all the methionine positions (Frank et al., 1985). This fact may enhance our understanding of the biological pathways in accessing selenium reduction.

One of the major strategies of bacterial resistance to heavy metals had earlier been noted to be the development of efficient efflux systems (Wong and Wood, 1984). Detailed reviews on bacterial transport systems have been reported (Kaback, 1971, 1974; Boos, 1974). Data from these earlier studies suggest that efflux

exchange, and counterflow are useful strategies for studying permease turnover, because they can be utilized to delineate specific steps in the overall catalytic cycle (Kaback, 1986). Therefore, it is pertinent to have a clear and specific understanding of the kinds of proteins involved in metal uptake.

The binding-protein transport system, sometimes referred to as the periplasmic transport system is thought to be the mode of metal uptake by bacteria (Ames, 1986). Kaback (1984) in characterizing transport systems had reported the existence of two major classes of transport systems, based on their response to a physical treatment, osmotic shock. The mechanics of this process, along with an abundance of information on the nature and properties of the outer membrane have been illustrated (Nikaido and Vara, 1985). It is significant to note that the metal-binding proteins were sensitive to osmotic shock treatment (Kaback,1986). During the process of osmotic shock treatment, a special class of proteins is released into the medium, called the periplasmic proteins. The term "periplasmic proteins" should be taken more as an operational definition than as an indication of a physical location, since a protein, such as, EF-TU (protein elongation factor), which must be functioning well inside the cell, actually is released by a treatment very similar to osmotic shock (Jacobson et al., 1976; Ames and Nikaido, 1979). On the other hand, a number of phosphatases, which are typical periplasmic

enzymes, as far as their functions are concerned, are poorly released by osmotic shock (Kier et al., 1977). One of the many goals of environmental microbiologists is to be able to characterize specific transport systems for individual metals. Presently, this goal has been elusive, with the exception of mercury. The evidence for a mercury-specific transport system and its role in Hg^{++} resistance were first reported by Foster et al. (1979) and Nakahara et al. (1979). One of the difficulties encountered in characterizing the mercury transport system, was the absence of titratable thiol groups (Nakahara et al., 1979). This problem may well account for the difficulties in having an up to date transport system for most of the highly toxic metals such as lead, chromium and selenium.

CHAPTER III

MATERIALS AND METHODS

Simulated Pond Design

Figure 1 illustrates the simulated pond used in this study. Clear plastic tanks (25 cm x 15 cm x 12 cm) were layered with 3 kilograms of sandy-loam soil, collected from Savannah, Georgia. To maintain a consistent soil bed, wire gauze was placed on top the soil, followed by a layer of oyster shells, which provided a natural buffering base for the aqueous environment. Metal solutions were made with three liter of culture media (brackish water), made up of the following salts in grams/liter: $CaCl_2$ 0.07, NaCl 0.35, $MgSO_4$ 0.06, KCl 0.03, and K_2HPO_4 0.5. The ecosystem was maintained at 30 ° C in a controlled Environment chamber, model 845 (New Brunswick Scientific, Edison, NJ.) Illumination was provided on a day/night cycle by 3 incandescent 60-watt bulbs and 2 florescent 34-watt tubes, placed 25 centimeter from the pond surface.

Development of Metal-tolerant Mixed Microbial Strains

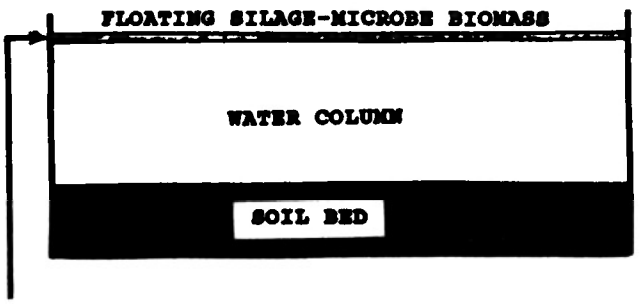
A mixed microbial adaptation development ultra-assay (MADU) was employed for the development of metal-tolerant microbes. The procedure is described as follows:

1. Set up mixed microbial culture in a 3-liter brackish

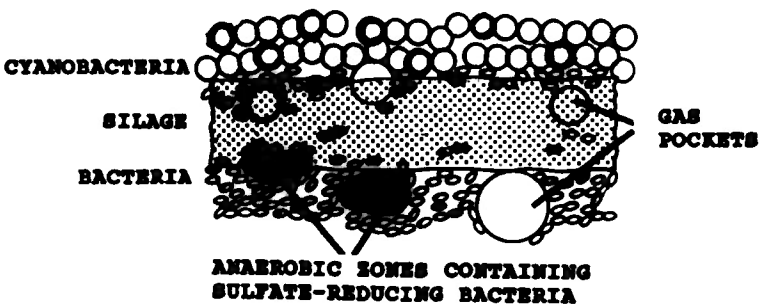
Fig. 1. Illustration of the simulated pond design. The pond is made of three basic components:

1. The floating mixed microbial biomass.
2. The water column.
3. The soil bed.

SIMULATED POND



MODEL OF FLOATING BIOMASS:



media, containing the maximum inhibitory concentration of each metal of choice.

2. Induced metal tolerance in the mixed cell population by allowing culture to age in the metal culture for an initial period of 30 days.
3. Resuspended viable microbes in the aged culture by adding 500 ml of metal solution (100 ppm). This step may vary depending on the toxicity of the metal on the original mixed microbes. This step was known as the dosing period.
4. Aged culture for additional 7-10 days.
5. To show that viable microbes were metal-tolerant than the original mixed microbes, (and not due to factors such as, error in inoculation, media preparation or assay), selected mixed microbes were tested for the following:
   i. Total viable cells were visualized on nutrient agar or on nutrient agar supplemented with Allens' media (Allens and Arnon, 1955), for bacteria and cyanobacteria respectively. A logarithm bacterial population of 5.0 was indicative of the presence of viable bacterial cells. While the presence of heterocyst within the vegetative cells of the cyanobacteria was indicative of nitrogen-fixing cyanobacterial cells.
   ii. Metal uptake dynamics in simulated pond systems as earlier described. This included the

monitoring of the following phases: (1) bacterial, (ii) cyanobacterial, and (iii) mixed microbial mat formation. A shorter phase in each case suggested the presence of metal-tolerant strains. (iv) Percent metal recovery from the mixed microbial mat; a higher metal recovery also suggested increased metal-tolerance in the microbes.

6. Repeated stages 3-4 through several cycles until desirable microbes were obtained.

Bacterial Strains

Bacterial strains constituted those isolated from the soil bed and water column of the simulated pond. In order to ensure optimum microbial growth, and a stable microbial mat, bacterial strains were adapted for increased metal tolerance as described earlier.

Cyanobacterial Strains

The cyanobacterial strains used in this study were non-axenic strains of Anaebena spp, NB-19, isolated and adapted for metal tolerance (Table 1). Microscopic view of strains reveal that all are filamentous and heterocyst-forming cyanobacteria. The standard inoculum for all metal uptake experiments was 0.04 mg/tank.

TABLE 1. TOLERANCE LEVELS OF METAL-ADAPTED MICROBIAL STRAINS

CYANOBACTERIAL LEVELS

STRAINS	METALS	TOLERANCE ADAPTATION LIMIT (ppm)		
		BEFORE	AFTER	FUNCTIONAL RELEVANCE
NB-19-Pb	Pb	30	900	500
NB-19-Cr	Cr	25	150	100
NB-19-Se	Se	25	80	50
BACTERIAL LEVELS				
MRB-Pb	Pb	100	900	500
MRB-Cr	Cr	25	300	100
MRB-Se	Se	25	150	50

NB-19-metal = metal-adapted cyanobacterial strain, adapted to the metal indicated.

MRB-METAL = multiple resistant bacteria, adapted to the specific metal indicated.

Source of Silage

Silage was prepared according to the procedures of (Mchan, personal communications), with minor modifications as described below:

(a) Cut green grass to 1/4-1/2 centimeter in sizes.
(b) Tightly packed the grass clippings in 1-liter jars to exclude air pockets.
(c) Cultured anaerobically for 20 days at room temperature.
(d) Good silaged grass was detected by the smell of vinegar.

Heavy Metals

Specific metals studied were spectrophotometric grades of lead, chromium and selenite (Fisher Scientific, Norcross, Ga.). Appropriate concentrations of each metal were prepared by diluting standard solutions in 3-liters of culture media, or on the protocol recommended for atomic absorption spectrophotometry.

Sampling and Analyses

Mixed microbial biomass was carefully harvested by lifting the wire gauze from the soil bed of the simulated pond followed by a manual racking of the floating mat. Water samples were collected at a depth of 2 centimeter and soil samples taken as random cores, also taken at depth of 2 centimeter, below the soil surface. All test samples were acid hydrolyzed (Standard methods of water and wastewater examination, 1985), and analyzed for metal

content with a Varian 775 atomic absorption unit (Varian Instrument group, Sugarland, Texas). For experiments designed to show bacterial involvement in metal mobilization, five milliliter of water sample were collected and passed through the millipore filter (0.45 µm), filtered and unfiltered samples then assayed for metal content.

Acid Hydrolyses

Test samples were acid hydrolyzed (Standard methods for water and wastewater examination, 1985) with minor modifications as described below:

(a) Measured test sample into 250 milliliter Erlenmeyer flask.
(b) Acidified sample with 20 milliliters of 70% nitric acid and evaporated to a small volume.
(c) Continued evaporation to near dryness, taking precaution to prevent spattering.
(d) Completed acid hydrolyses by adding 10 milliliters of 50% hydrogen peroxide.
(e) Finally, brought hydrolysate to original volume by adding deionized distilled water.

Atomic Absorption Spectrophotometry

The hydrolysates obtained from acid hydrolyses were assayed for metal content with the atomic absorption unit (Varian Instrument group, Sugarland, Texas). The attenuation of the light beam passing through the sample was logarithmically related to the concentration of the metal atom being measured. This process was achieved by

passing the test sample in a controlled acetylene flame, connected to the nebulizer, where atomization of the metal ions took place. Consequently, the light beam was separated by a monochromator and the absorbed metal atom, detected with a photomutiplier. The following wavelengths were characteristic of each metal tested: lead 217 nm, chromium 359.7 nm, and selenium 198.7 nm.

Determination of The Role of Bacteria In Metal Mobilization

Scanning electron microscopy and micro-x ray analyses. In order to correlate the association between adapted microbes and the metals in culture, bacteria and cyanobacteria were collected from the simulated pond during their respective blooms. Samples were then air dried in a dust-free environment and fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. The specimens were dehydrated in a graded series of alcohol (30, 50, 70, 90, and 100%), critical point dried with liquid carbon dioxide and sputter coated with gold/palladium. In order to correlate the presence of metals with test samples, each sample was carefully mounted upon carbon stubs and examined by scanning electron microscopy (ETEC OMNISCAN, Kevex corporation, Foster city, Ca.). Subsequently, samples were assayed with X-ray energy spectrophotometer (Kevex micro-x 7000 analytical spectrophotometer, Kevex corporation, Foster city, Ca.) for the quantification and identification of the metals (or the metalocompounds) by peak heights.

Bacterial Metal Chelation

To determine if metals were chelated in the media by extracellular proteins, the spent media derived after bacterial culture were concentrated 100 times by filtration, using an Amicon B15 concentrator (Grace and Company, Denvers, Ma.). Concentrated samples were tested for metal and protein, using an atomic absorption unit and a 280 nm absorbance using a UV-spectrophotometer, respectively. Specific proteins were examined by native polyacrylamide gel electrophoreses. In order to correlate specific proteins with metals, individual protein bands were cut out from the gel and tested for metal content.

Bacterial Metal Adsorption/Absorption

Metals not complexing with extracellular protein were determined by a differential centrifugation of the total bacterial lysate, in order to obtain membrane and cytoplasmic fractions. This procedure (Osborn, 1972) is described below:

1. Harvested bacterial cells from one hundred milliliters of culture by centrifugation at 10,000 rpm for 10 minutes at 4° C.
2. Resuspended pellet in TE-B (10 mM Tris, 5 mM EDTA, 1 mM 2-mercaptoethanol) buffer and centrifuged at 4,000 rpm for 3 minutes, to remove cellular debris.
3. Lysed cells by sonication in ice 3 times for 15 seconds intervals; separate unlysed cells.

4. Centrifuged total lysate at 28,000 rpm (100,000 xg in a SW50 rotor) for 1 hour to separate cell membranes from cytoplasmic components.
5. Determined and identified both metal and protein in membrane (pellet) and cytoplasmic (supernatant) fractions atomic absorption unit and SDS-PAGE analyses as described below:

Protein Concentration Assay

The quantitative protein determination was performed according to the Bio-Rad protein assay. Here, several dilutions of protein standard (Bovine serum albumin, BSA) containing from 0.2 to 1.4 mg/ml were prepared. In this assay, 0.1 ml of standards containing appropriately diluted samples were placed in clean dry test tubes and 0.1 ml of sample buffer made in 0.1 M Tris-HCl and 0.01 EDTA at pH 8.0 was placed in a "blank" test tube as a reference. Five milliliters of diluted (1:4) Bio-Rad dye reagent was added to each sample, mixed gently, and allowed to stand for a period of 5 to 15 minutes before the absorbance at 595 nm was determined. The optical density versus concentration of standards was plotted and protein concentrations of samples were determined by recording absorbance relative to the standard curve.

Identification of Metal-binding Proteins by polyacrylamide Gel Electrophoresis (PAGE)

Native polyacryalamide gel electrophoresis. Native-polyacrylamide gel electrophoresis (N-PAGE) was performed

by the method of Laemmli (1973) with linear gradient gels of 5 to 15%. Protein samples were prepared by heating at 100° C for 2 minutes in sample buffer (1 mM 2-mercaptoethanol, 10% [vol/vol] glycerol, 1 mM Tris, 0.5% bromophenol blue, pH 6.8). Separation of protein samples was performed using gels containing 30% acrylamide and 0.8% bisacrylamide. Gel thickness varied from 1.0 mm to 2.0 mm.

Prior to adding protein samples to the gel, samples containing membrane fractions were treated with 5% (wt/vol) Brij 58 (polyoxyethylene cetyl ether) and Triton X-100 (Sigma Chemical Co., St Louis, Mo.) in a 1:1 ratio. (Myers et al., 1987). Fifty micrograms of each protein sample were applied to the gels. Gels were electrophoresed at 25 milliamps for 5 hours or until the tracking dye was about 3 to 5 centimeter from the bottom of the gel. After electrophoresis, the gels were stained with an aqueous solution containing 0.25% (wt/vol) coomassie brilliant blue (Bio-Rad Laboratories. Richmond, Ca.), 50% (vol/vol) methanol, and 10% (vol/vol) acetic acid. Gels were destained in 40% methanol/12% acetic acid solution. In some cases, proteins were silver stained (Switzer et al., 1979).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS-PAGE was performed similarly as described above (Native-PAGE) in sample buffer which in addition, contained 10% SDS. Fifty micrograms of each protein sample and 10 micrograms of standard molecular weight

proteins (Pre-stained SDS-PAGE standards, Bio-Rad Laboratories, Richmond, Ca.) were applied to the gels as earlier described. Gels were electrophoresed in Laemmli (1973) electrophoresis buffer containing 10 x Tris glycine, 20% SDS and distilled water. Gels were similarly destained and stained as earlier described (Native-PAGE).

Identification of a Chromium Reductase

Determination of spectrophotometric wavelength. Samples of hexavalent chromium ions before and after bacterial culture were scanned at wavelengths ranging from 200-700 nm, using a DU-7 spectrophotometer. Absorption profiles determined the wavelengths at which to assay hexavalent and reduced trivalent chromium ions.

Ion exchange chromatography. Five milliliters of a chromium-adapted bacteria lysate was passed through a 0.7 x 12 centimeter diethminoethlaminoethyl (DEAE) sephacel, containing 10 milliliters of potassium dichromate (hexavalent chromium). In order to allow time for fixation (reduction of hexvalent chromium), the column was allowed to stand for 1 hour. One hundred 3 milliliter fractions were collected and assayed for protein, and chromium reduction activity; reduction activity involved reading samples with the spectrophotometer at 418 nm, the wavelength that expressed maximum absorption of trivalent chromium.

Quantitation of Trivalent Chromium

In order to correlate fractions with trivalent chromium, two separate assays were performed: total chromium and hexavalent chromium analyses.

Total chromium assay. To assay for total chromium, samples were acid hydrolysed and then tested for total chromium with the atomic absorption. Refer to the section on "Atomic absorption spectrophotometry" for details.

Test for hexavalent chromium. Measured volumes of standard dichromate solution (5 g/ml) ranging from 2.00 to 20.00 milliliters, to give standards for 10 to 100 g Cr were set up. A solution of 1,5-diphenylcarbazide was prepared by dissolving 250 mg of the salt in 50 milliliters of acetone. To each of the standard hexavalent mix, 2 milliliters of diphenylcarbazide solution was added. Samples were allowed to stand for 5 to 10 minutes for full color development. Appropriate portions of each mix were transferred to a 1 cm absorption cell and absorbance measured at 540 nm. Absorbance readings obtained were used for preparing a standard hexavalent chromium curve. Hexavalent chromium in the samples was determined from the standard curve.

The differences obtained from total chromium measurements (using atomic absorption) and the hexavalent diphenylcarbazide assay gave the amounts of trivalent chromium in the fractions. From these readings, percentage of trivalent chromium was computed.

Physicochemical Characterization of the Mixed Microbial Mat

A micro-analysis to determine the physical properties of the mixed microbial mat was performed as outlined below:

Dissolved oxygen. Dissolved oxygen was determined using M1-730 micro-oxygen probe (Microelectrodes, Londonderry, N.H.), with a typical sensitivity of 12 pico amps/$Mm0_2$, relayed at a response time of less than 20 seconds. Values of dissolved oxygen in the system were determined from a standard, calibrated with a 0, 21, 100% oxygen gas.

Oxidation-reduction potential (ORP). The ORPs of the microenvironments in the mixed microbial mat were measured with a M1-710 ORP microprobe (Microelectrodes, Londonderry, NH). These measurements were performed by probing randomly at the top, and beneath surfaces of the mat.

Analyses of anaerobic zones. Results obtained from the dissolved oxygen and ORP analyses were used for the determination of the presence of anaerobic zones within the mat structure, since anaerobic conditions correlate indirectly and directly to oxygen and ORP, respectively.

Confirmation of anaerobic zones. Presence of anaerobic environments was confirmed by the presence of viable anaerobes, including sulfur-reducers, cultured on API agar (Difco Laboratories, Detroit, Michigan). Presence of black colonies was indicative of the presence

of sulfur-reducers. Evidence of lead sulfide precipitation was further verified with lead acetate indicator paper.

Statistical Analyses

Each experiment was repeated at least three times. Statistically significant differences were sought by an analyses of variance (ANOVA) using the Student-T-test (Daniel, 1983).

CHAPTER IV

EXPERIMENTAL RESULTS

This research was initiated with a series of studies which determined the background parameters for the conclusion of this study. The latter studies included:

(1) The design and construction of the simulated pond (Fig. 1).

(2) Strain development for metal tolerance.

Formation of Microbial Biomass and Metal Mobilization in Simulated Ponds

The events that led to the formation of the mixed microbial mat and mobilization of heavy metals typically resulted in three distinct microbial phases:

(i) A bacterial phase, which was characterized by the mobilization of metal ions and their transport from the soil bed to the water column.

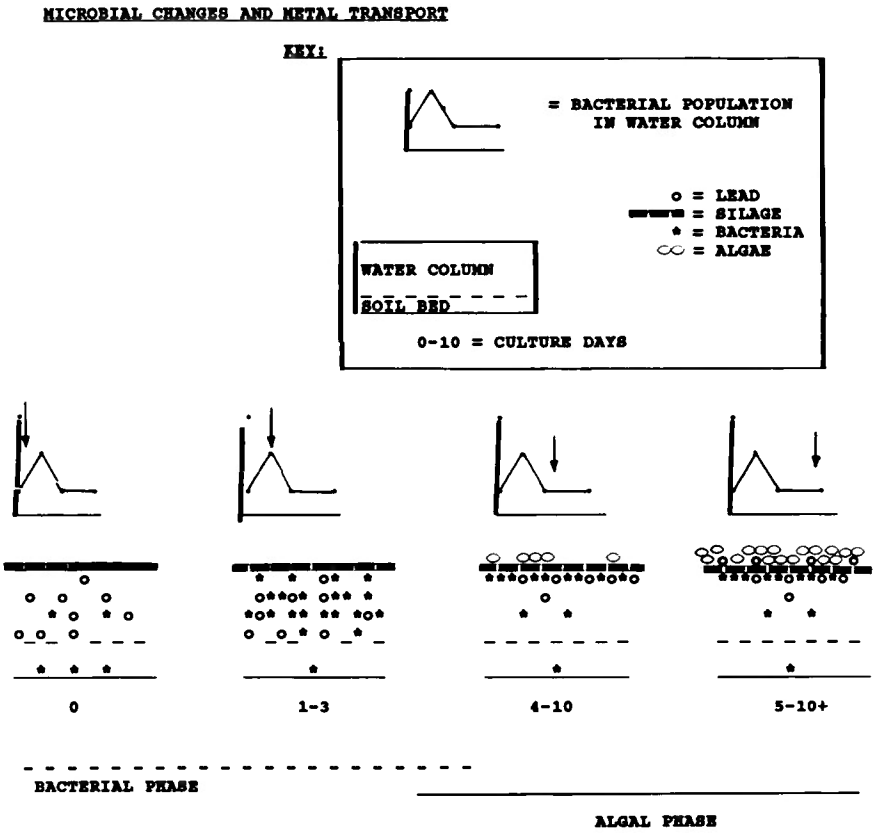
(ii) The cyanobacterial phase, a period marked by the complexation of the mobilized metal ions to solid and aqueous phases.

(iii) The establishment of the microbial mat, during which the complexed metal ions were stabilized within the matrix of the mat.

The description of the microbial successions are described in the list of events represented in (Fig. 2).

Fig. 2. Description of the different microbial phases and metal transport in the simulated pond.

MICROBIAL CHANGES AND METAL TRANSPORT



Apart from the mobilization and transport of the metal ions which occurred during the bacterial phase (0-3 days) the period was also marked by high bacterial counts (10^7 - 10^9 cell/ml). Following the bacterial phase, was the cyanobacterial stage. This stage was dependent upon the success of the initial bacterial phase, and characterized by the formation of algal bloom, which appeared as a green network of filamentous algal strains. Following the cyanobacterial stage was the formation of the mixed microbial mat. The generated mixed microbial mat represented the last stable microbial phase in the simulated pond. Its formation (microbial mat) was characterized by a complex matrix of cyanobacteria, grass silage, and a heterogenous population of bacteria. As shown on figure 1, the entire mat was held buoyant at the pond surface by the presence of gas pockets, which are formed within the mat complex.

Another important feature of the mixed microbial mat, was the presence of anaerobic zones. One of such zones is depicted in the block shown on the electron micrograph (Figure 3). On analyses, the anaerobic region was found to contain sulfate-reducing bacteria. Evidence of sulfate-reducers, was shown by the presence of black colonies isolated on API agar (data not shown).

Fig. 3. Scanning electron micrograph of the mixed microbial mat.

The marked rectangular region shows an area of anaerobic activity. A gross observation of the picture shows presence of a heterogeneous mixture of bacteria, filamentous algae and silage.

Some evidence has been developed regarding the chemical environment of the mat, which suggested that the presence of reducing conditions are major controlling factors in the behavior of the metals in the simulated ponds. Table 2 showed the results of dissolved oxygen (DO) and oxidation-reduction potential (ORP) analyses of the mat. As can be seen, there were significant differences between the top and bottom mat. Micro-x-ray analyses of the mat, using x-ray energy spectrophotometer, revealed specific peaks, which corresponded to Pb and PbS (Figure 4 a and b), respectively. The other major peaks (Figure 4 c and d) represented Ca^{2+} and PO_4^{2-} ions, respectively. The smaller peaks are combinations of Mg^{2+}, Na^{+} and unidentified trace metals, which were components of the culture media.

Strain Development for Metal Tolerance

The mixed microbial adaptation development ultra-assay (MADU), described earlier, produced elevated metal tolerances in both bacteria and cyanobacteria (Table 1). The results from Table 1 indicated that after the adaptation process, there were 10, 6, and 3-fold increases in cyanobacterial-metal tolerance for lead, chromium and selenium, respectively.

TABLE 2. MICRO-PROBE ANALYSES OF MIXED MICROBIAL MAT FOR DISSOLVED OXYGEN (DO) AND OXIDATION-REDUCTION POTENTIAL (ORP) LEVELS

MAT	DO (mg/ml)	ORP(mV)
TOP	3.4 ± 0.3	-40 ± 1.3
BOTTOM	0.0 ± 0.0	-200 ± 2.4

Values represent the means and standard deviations of 9 separate experiments. Each analyses of DO and ORP was performed by probing randomly at the top and beneath of the mat, using micro-probes of oxygen and ORP, respectively.

Fig. 4. Micro-X-ray profile of mixed microbial biomass.

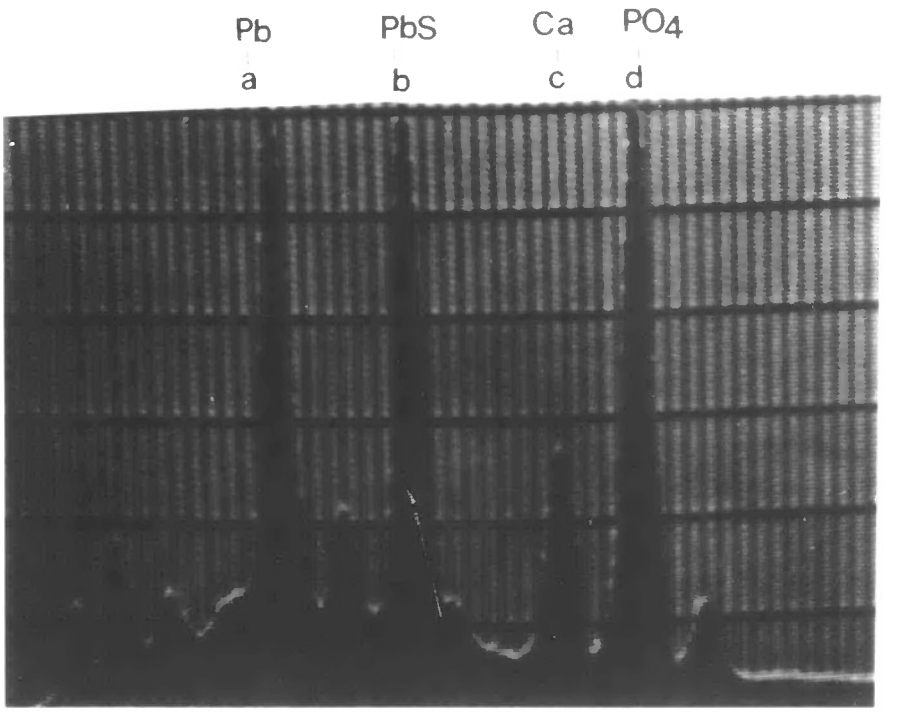
The major peaks illustrated are:

(a) Lead peak.

(b) Lead sulfide peak.

(c) Calcium peak.

(d) Phosphate peak.


Pb
a
PbS
b
Ca
c
PO4
d

In the bacterial-metal tolerance, the change in the tolerance level after adaptation were 9, 12, and 6-fold increases for lead, chromium and selenium, respectively.

Since the development of the cyanobacterial phase was dependent on the success of the initial bacterial phase, a functional relevance concentration was determined for each metal. From Table 2, the functional relevance concentrations which supported both the cyanobacteria and bacteria in lead, chromium and selenium infusions were 500, 100, and 50 parts per million (ppm), respectively.

Uptake Dynamics of Lead in the Simulated Pond

Figure 5 shows the results produced from a series of 10 identical simulated ponds, inoculated with lead-tolerant microbes and 300 mg/tank of lead. Daily analyses of filtered and non-filtered water samples, and the microbial biomass showed that 40-45% of the lead ions were associated with bacteria.

The first 4 days was characterized by a bacterial bloom of 10^7 to 10^9 cells/ml. As can be seen from figure 5, during the bacterial bloom (0-4 days), about 50-60% of the lead ions lost from the water column were periodically found at the soil bed and back to the water column. However, at the beginning of the cyanobacterial stage (4-7 days), the lead ions began to accumulate in

Fig. 5. Uptake dynamics of lead in a mixed microbial system.

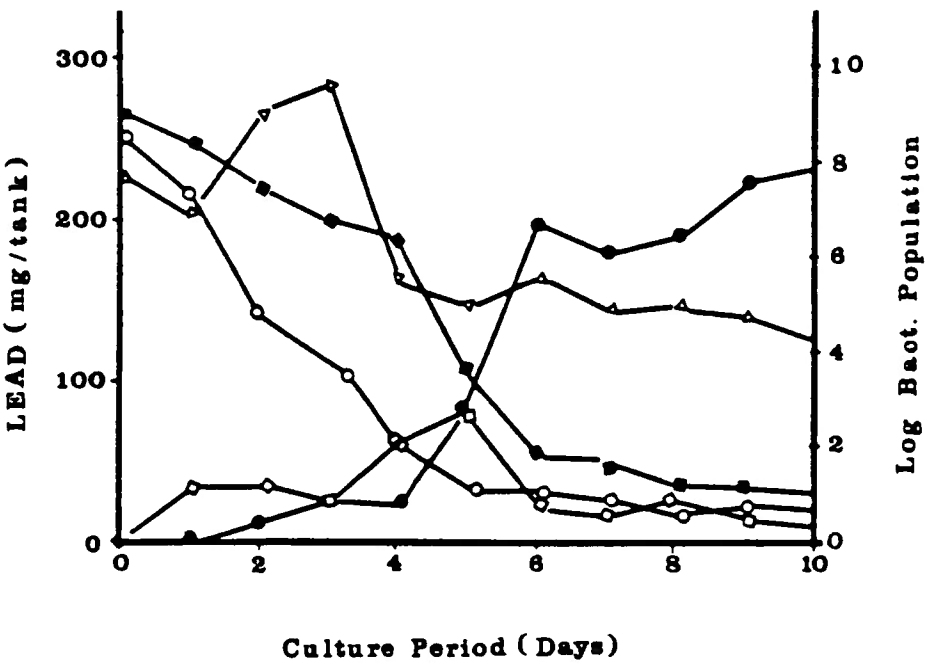
(▲) Log bacterial population.

(■) Amount of lead in unfiltered water sample.

(○) Amount of lead in filtered water sample.

(●) Amount of lead in the microbial mat.

(□) Amount of lead in the soil.


300
200
100
0
LEAD (mg/tank)
0
2
4
6
8
10
Culture Period (Days)
10
8
6
4
2
0
Log Bact. Population

the surface microbial biomass. After 10 days of culture (when the tenth tank was harvested), the final analyses of the mixed microbial mat (dry weight, 4.731 grams) indicated that 83% of the lead were recovered from the mat. This value was equivalent to 41.77 mg Pb per gram biomass.

Loading Capacity of Lead in the Simulated Pond

In order to test the holding capacity of the mixed microbial system for lead uptake, a 40-day lead spiking experiment was maintained. Figure 6 shows the result of this experiment. Lead was spiked 9 times into the system at a concentration of 300 mg/spike. The first spike was added at day 8, after 70% of the initial lead was removed from the water column.

Thereafter, additional lead spikes were performed 2-3 days after the preceding spike. The experiment was stopped when it showed that the lead content in the soil was increasing and also due to the deterioration of the integrity of the surface mat. At the end of the experiment, a total of 2700 mg of lead had been added to the system. The analyses of the resulting mixed microbial mat (dry weight, 11.15 grams) indicated that 1620 mg or 60% of the lead was recovered from the mixed microbial mat. This was equivalent to 145.3 mg Pb per gram mat biomass. Thirty and 5 percent of the lead ions remained in the soil bed and water column, respectively.

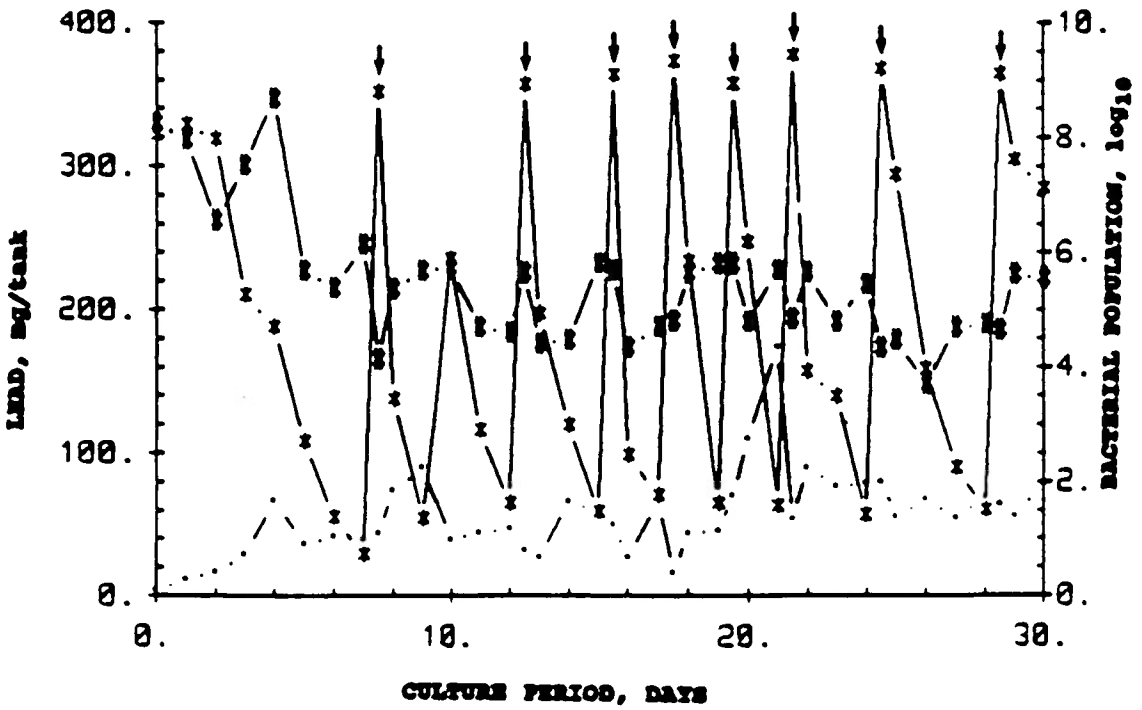
Fig. 6. Loading capacity of lead in the simulated pond.

(x) Lead in the water column.

(•) Lead in the soil.

(#) Log bacteria.

(▾) Lead spikes.


400.
300.
200.
100.
0.
10.
8.
6.
4.
2.
0.
0.
10.
20.
30.
LEAD, mg/tank
BACTERIAL POPULATION, log10
CULTURE PERIOD, DAYS

Lead Speciation in the Simulated Pond

The mobilization and transport of metal ions in aquatic systems is thought to be controlled by the prevailing metal species present in the system (Shaw and Alfred, 1970). In aquatic environments, metal ions are known to form various metal species, based on the composition and prevailing pH of the system (Sillen and Martel, 1971). Figure 7 represents the predominance area diagram based on the composition of lead ions in the water column, using solubility products compiled from Sillen and Martel (1964, 1971). Based on the observed range of pH conditions (7.5-9.9) in the system, the predominant solid phase of lead was concluded to be basic lead chloride, $Pb_2Cl(OH)_3$ (Fig. 7).

Uptake Dynamics of Chromium in the Simulated Pond

Figure 8 shows the results of chromium uptake in the system utilizing the chromium adapted bacteria and algal strains. During the 9-day culture period, the bacterial bloom occurred within 10 hours of culture, which was 62 hours faster than the general period, typical of lead cultures.

The above observation was significant in explaining the rapid reduction of hexavalent chromium in the system. It (the rapid bacterial bloom) also suggested the active bacterial role in chromium uptake in the simulated pond.

Fig. 7. Predominance area diagram of lead species in the simulated pond.

(X) Predominant solid lead species in the simulated pond.

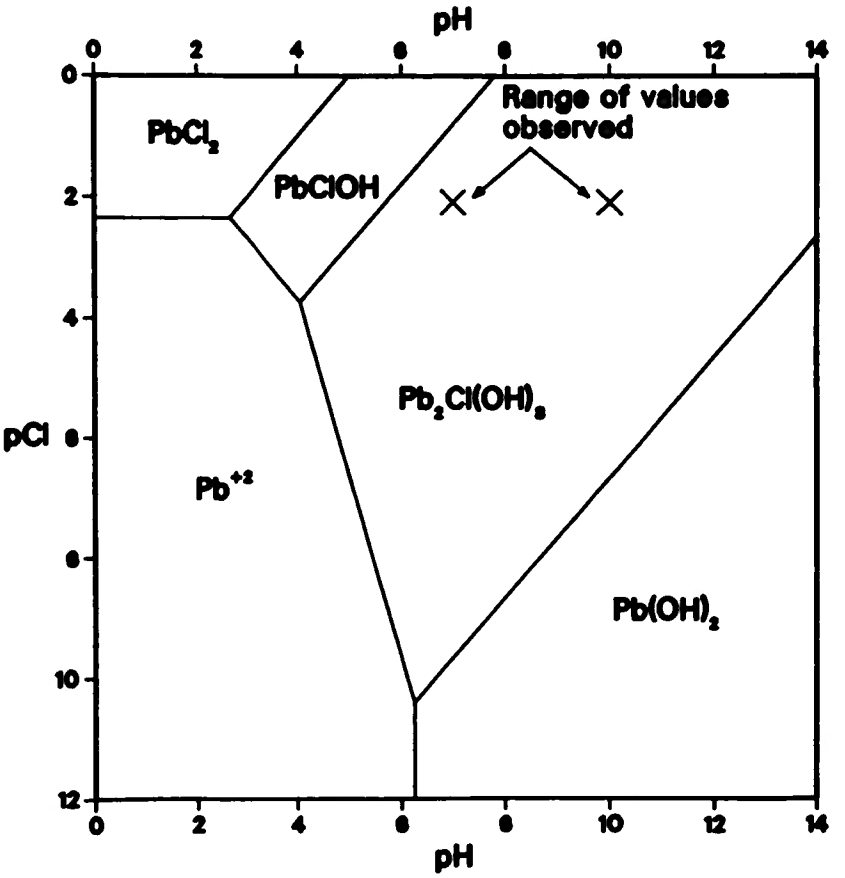

pH
0
2
4
6
8
10
12
14
pCl
$PbCl_2$
PbClOH
Range of values observed
$Pb_2Cl(OH)_3$
Pb^{+2}
$Pb(OH)_2$

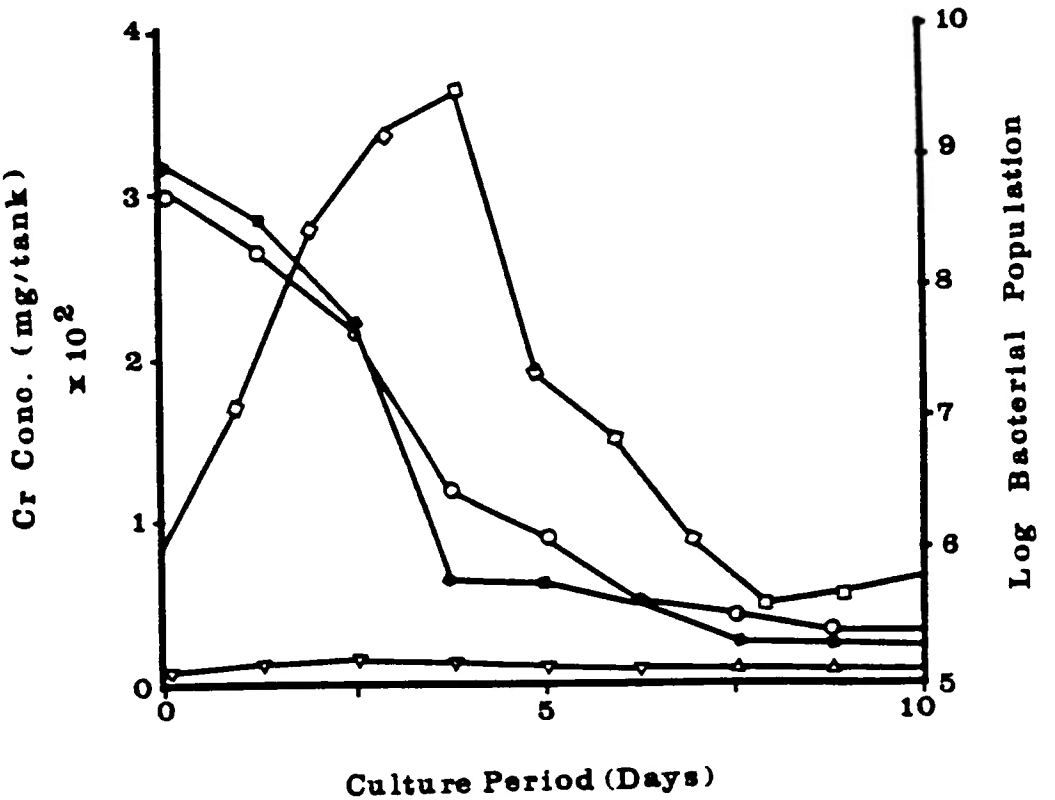
Fig. 8. Uptake dynamics of chromium in the simulated pond.

(□) Log bacteria population.

(■) Amount of chromium in the unfiltered water sample.

(o) Amount of chromium in the filtered water sample.

(Δ) Amount of chromium in the soil.


Cr Conc. (mg/tank)
x 10²
Log Bacterial Population
Culture Period (Days)
0
1
2
3
4
5
6
7
8
9
10
0
5
10

The cyanobacterial or algal stage formed after 30 hours (90 hours faster than previously recorded for lead cultures). It was interesting to note that before the beginning of the algal phase, at day 3, 99% of the hexavalent chromium had been mobilized and transformed by the bacteria to the trivalent state. This was evidenced by the separate green layer of chromium hydroxide formed on the oyster shells beneath the floating microbial mat (data not shown). The final level of trivalent chromium in the mat (dry weight, 5.37 gram), was 250 mg or 84%. This represents 46.6 mg Cr per gram biomass. Trivalent chromium levels remaining in the soil and water column, were 3 and 9%, respectively. Four percent of the chromium was unaccounted for.

Loading Capacity of Chromium in the Simulated Pond

Figure 9 shows the results of experiments designed to test the efficiency of the system for its holding capacity of chromium. During the 21-day culture period, a total of 1500 mg were added to the simulated pond, at a rate of 300 mg Cr/spike. Similar to the lead spiking experiments, the additional chromium spikes were added after an average removal of 70% of the chromium at days 6, 9, 12, and 16. As can be seen (Fig. 9), the older the culture, the lesser time it took to remove the chromium.

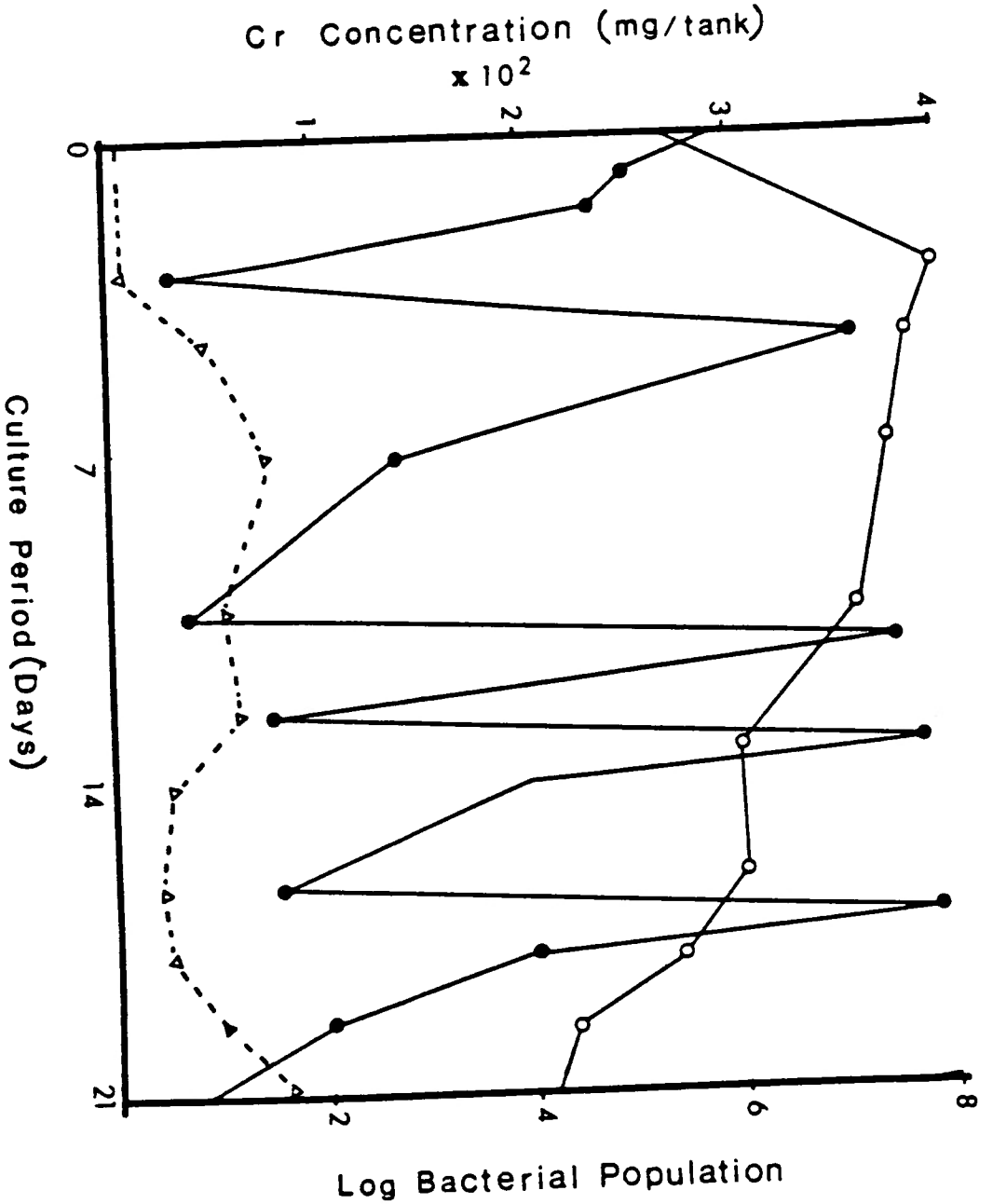
Fig. 9. Loading capacity of chromium in the simulated pond.

(●) Chromium in water column.

(Δ) Chromium in the soil.

(O) Log bacteria population.

(●) Chromium spikes.


Cr Concentration (mg/tank)
x 10²
1
2
3
4
0
7
14
21
Culture Period (Days)
2
4
6
8
Log Bacterial Population

The rapid removal of the chromium ions corresponded to the emergence of chromium-tolerant bacteria in the system. Experiments were stopped when the mat (biomass) showed either deterioration or blanching, as evidenced by a rapid loss of its green coloration. The pH range during the 21-day period of culture was 7.5-8.3. A total of 1050 mg of chromium were finally recovered from the biomass (dry weight, 6.89 grams), which was 152 mg Cr per gram biomass.

Chromium Speciation in the Simulated Pond

Removal of heavy metals by precipitation had earlier been documented (Porter et al., 1971). The precipitation process, which may involve the initial reduction of the metal in low pH ranges by the use of flocculating agents, have been noted to be an efficient method for the removal of heavy metals from wastewater (Christensen and Delwiche, 1981).

Equilibrium constants used in solubility calculations of chromium as $Cr(OH)_3$ indicated that the pH of minimum solubility for the trivalent chromium was 8.2 (Paterson et al., 1977). In course of these experiments, there was evidence to show that the observed pH values (6.9-8.3) were in agreement with theoretical values. This was found to be adequate for the reduction of hexavalent chromium to the trivalent state, which supported the formation of chromium hydroxide as the predominant solid species in the simulated pond. As earlier reported

(loading capacity experiments), the evidence for the presence of chromium hydroxide was seen as a layer of green precipitation, formed beneath the floating microbial mat. Figure 10 shows the predominance area diagram, indicated by chromium hydroxide as the dominant chromium species.

Uptake Dynamics of Selenium in the Simulated Pond

Figure 11 represent the results of a 23-day culture in selenium-infused medium (150 mg/tank). Uptake dynamics were similar to those of lead and chromium, in that the mobilization of selenium by bacteria and the final deposition in the floating biomass, followed the same pattern. However, unlike lead and chromium, a prolonged lag phase (0-9 days) was observed. The latter period corresponded with a low bacterial population (10^3 - 10^4 cells/ml). Subsequently, between days 11-15, a bacterial bloom was observed (10^5 - 10^6 cells/ml), which corresponded with the mobilization of the selenium from the soil to the water column. An algal phase occurred between days 16-20. This phenomenum was also different from the succession of microbial phases seen in lead or chromium. Analyses of the final biomass, dry weight, 3.63 grams, indicated that 38% (15.7 mg Se/gram biomass) were recovered from the mat. Forty and 20% of the

Fig. 10. Predominance area diagram of chromium species in the simulated pond.

(↓) Experimental pH values indicated that the predominant chromium species during uptake in the simulated pond was chromium hydroxide $Cr(OH)_3$

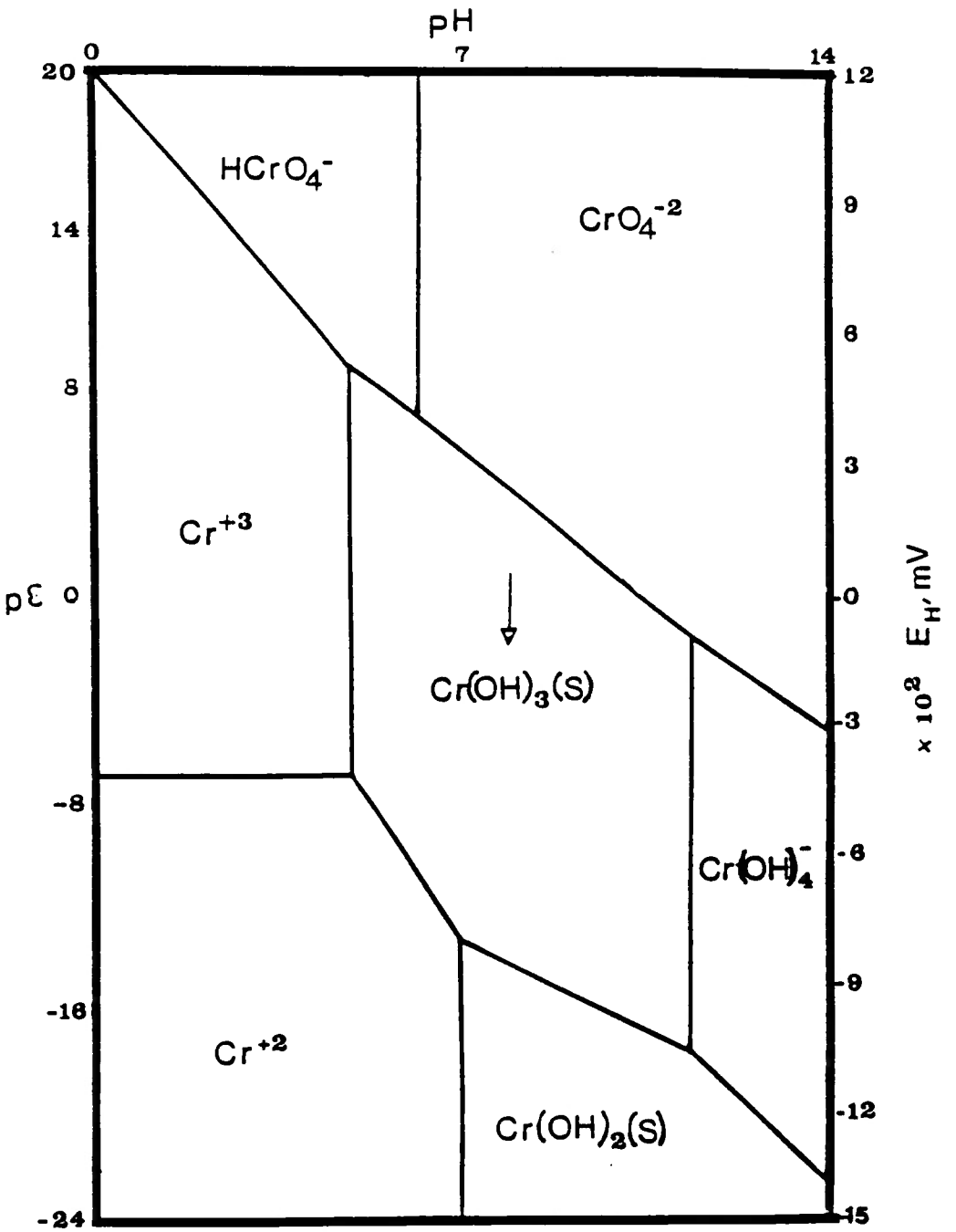

pH
0
7
14
20
14
8
pε 0
-8
-16
-24
12
9
6
3
0
-3
-6
-9
-12
-15
x 10² E_H, mV
HCrO4⁻
CrO4⁻²
Cr⁺³
Cr(OH)3(S)
Cr(OH)4⁻
Cr⁺²
Cr(OH)2(S)

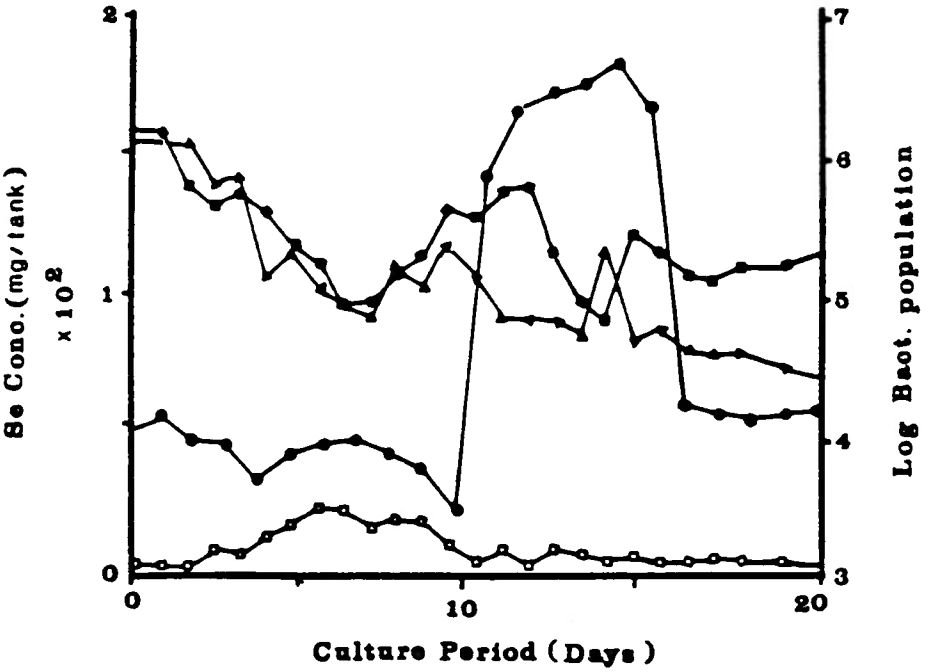
Fig. 11. Uptake dynamics of selenium in the simulated pond.

(●) Log bacterial population.

(■) Selenium in unfiltered water sample.

(▲) Selenium in filtered water sample.

(□) Selenium in the soil.


Se Cono.(mg/tank)
x 10²
Log Bact. population
Culture Period (Days)
0
1
2
3
4
5
6
7
0
10
20

selenium remained in the water column and soil, respectively. Due to the high toxity of selenium to themicrobes, no spiking experiments were performed.

Selenium Speciation

In order to correlate the pH values (7.5-8.8) observed during uptake and the predominant selenium species, that may exist in the simulated pond, the oxidation reduction potential (ORP) of the mat was determined. The measured ORP of the bottom mat was approximately -240 mV, in a pH range of 8.0-8.5. Using the experimental pH values, a predominance area diagram (Fig. 12) was determined. From figure 12, it showed that the chemical condition in the simulated pond existed for the reduction of the toxic selenite (+4 valency state) to the lesser toxic elemental selenium (0 valency state). The evidence for the presence of elemental selenium was observed as pink precipitates, deposited on the soil surface.

Determination of the Role of Bacteria in Metal Mobilization in the Mixed Microbial System

Figures 13 and 14 showed the results of experiments designed to correlate the association between lead-tolerant microbes and lead in lead-infused cultures. Using scanning electron microscopy, samples of bacteria and cyanobacteria, obtained during their respective blooms in the simulated pond, were examined. From figures 13 and 14, it was evident that both the bacteria

Fig. 12. Predominance area diagram of selenium species in the simulated pond.

(---) Based on ORP and pH values observed during selenium uptake, the chemical condition existed in the simulated pond for the precipitation of selenium as elemental selenium.

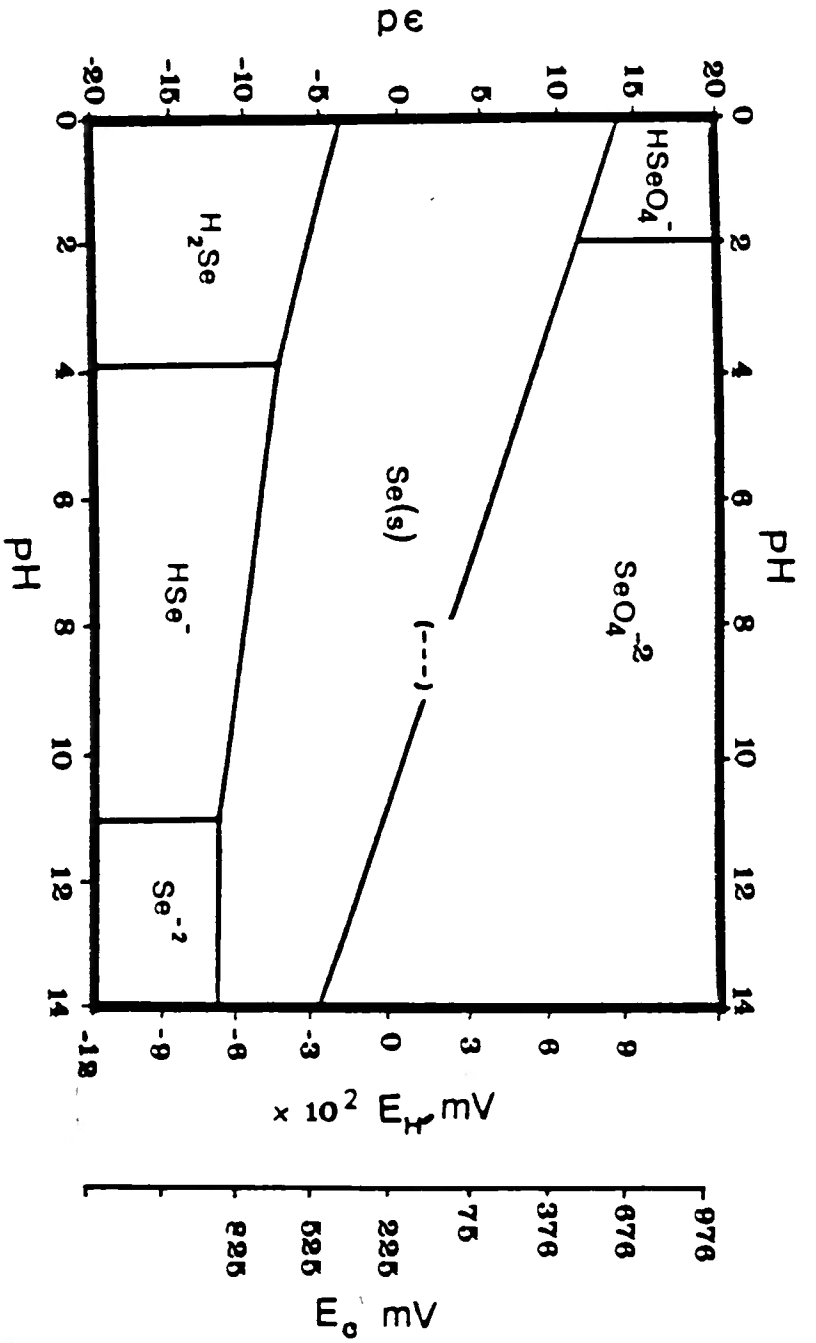

pε
pH
H_2Se
$HSeO_4^-$
HSe^-
Se(s)
SeO_4^{-2}
Se^{-2}
$\times 10^2$ E_H mV
E_o mV

Fig. 13. Association of lead-adapted bacteria to lead during lead uptake in the simulated pond.

(A) Dot map of lead ions.

(B) Scanning electron micrograph of bacterial strains isolated during bacterial phase. Comparison of panels (A) and (B) show strong correlations of the bacterial strains and the lead ions.

A

B

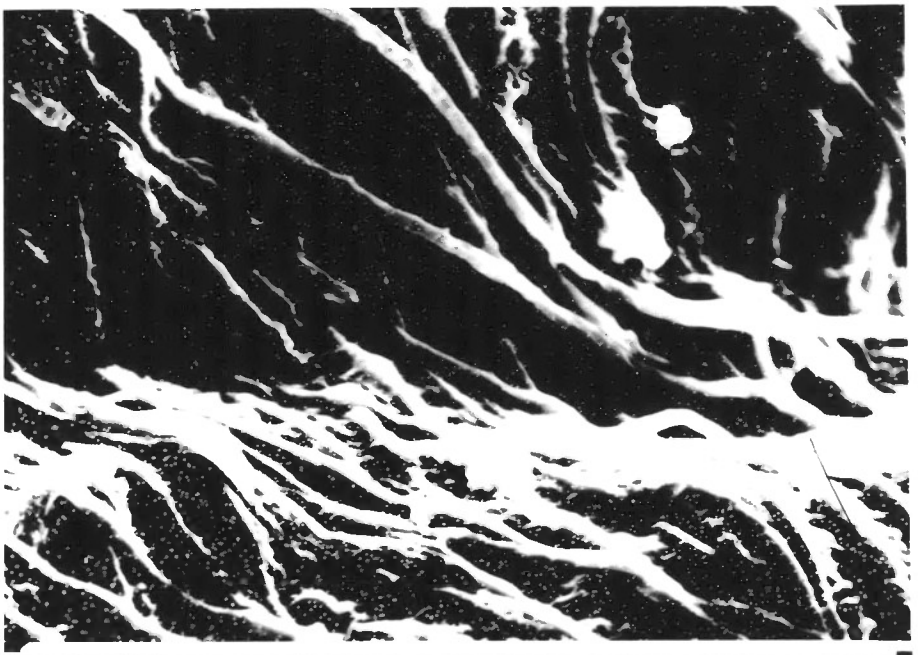
Fig. 14. Association of cyanobacterial-adapted strains to lead during lead uptake in the simulated pond.

(A) Scanning electron micrograph of mixed microbial mat showing cyanobacterial strains.

(B) Dot map of lead ions.

The electron micrograph indicated strong correlation between the cyanobacterial strains and the lead ions.

A



B

and the subsequent microbial mat (cynanobacteria and accompaning bacteria) strongly correlated to the lead dot map profiles. The data (Fig. 13) also showed that the majority of the bacteria were cocci, while the cyanobacteria in the microbial mat were filamentous and heterocyst-forming species (Fig. 14).

Metal Chelation

Since it was postulated, that one of the mechanisms of metal uptake by bacteria, may involve a chelating process, in which the metal ions were bound by some chemical moiety (Wong and Wood, 1984), experiments were set up to determine possible chelation of the metals by extracellular proteins. Atomic absorption analyses of concentrated media (after 96 hours of culture) before and after acid hydrolyses (Table 3), indicated differences on the amount of metals measured. The process of acid hydrolyses was designed to digest complexes that may bind to the metal ion being measured. The increased metal readings of media samples after acid hydrolyses may indicate the digestion of complexes that may have obscured the metal ions prior to acid hydrolyses. The results (Table 3) indicated that after acid hydrolyses 25, 20, and 2-fold metal increases were recovered from the media, containing lead chromium and selenium,

TABLE 3. METAL CHELATION IN CULTURE MEDIA.

METALS	METALS RECOVERED IN MEDIA			
	BEFORE ACID HYDROLYSES		AFTER ACID HYDROLYSES	
	mg	percent	mg	percent
LEAD	0.37 ± O.5	3.8	9.3 ± 0.5	93.0
CHROMIUM	0.48 ± 0.6	4.8	9.4 ± 0.1	94.0
SELENIUM	1.78 ± 0.2	34.0	3.4 ± 0.7	68.0

Values represent means and standard deviations of nine separate experiments. Spent media after 96 hours of bacterial culture in the presence of the above specified metals, were concentrated 100 times with B15 Amicon filter. Concentrated samples were assayed for the presence of the metals with the atomic absorption unit before and after acid hydrolyses.

respectively. In order to correlate the presence of metal ions to specific extracellular proteins, spent media from each metal assay, were concentrated, and analyzed on a native 5-15% polyacrylamide gel eletrophoreses (PAGE) (Fig. 15). Lane a represented the sample from a chromium-containing culture, which revealed the presence of a high molecular weight protein. Lanes b and c were concentrated samples from selenium and lead media, respectively. Both lanes (b and c) indicated the presence of low molecular weight proteins. Lane d was the control sample (culture media, containing metal-tolerant bacteria, without any metal). It was evident from lane d, that there was no extracellular protein in the media.

To determine if proteins observed in each of the lanes (Fig. 15) were associated with the respective metals, each lane was fractioned into different sections, and the different samples analyzed by atomic absorption for respective metal content. The atomic absorption results indicated that the high molecular weight protein (Fig. 15, lane a) exhibited the presence of chromium. No other sections of the lane (lane a) showed the presence of chromium. In lanes b and c none of the sections indicated the presence of selenium and lead, respectively, except for the lower molecular weight proteins pointed at both lanes (b and c). Analyses of the control lane (d), did not show metal association with any section of the lane.

Fig. 15. Native PAGE analyses of concentrated spent media for extracellular proteins.

(Lane a) Sample from a chromium-containing media, cultured in the presence of Cr-adapted bacteria.

(Lane b) Sample from a selenium-containing media, cultured in the presence of Se-adapted bacteria.

(Lane c) Sample from a lead-containing media, cultured in the presence of lead-adapted bacteria.

(Lane d) Control sample of a non-metal -containing media, cultured in the presence of metal-tolerant bacteria.


Lanes
a b c d

Distribution of Metals in Bacterial Subfractions

Lead. Part of the bacterial response to the detoxification of toxic heavy metals, may involve the adsorption or absorption of the metal ions to specific locations of the bacteria. Table 4 shows the results of experiments designed to examine the distribution of lead in lead-tolerant bacterial subfractions. The result (Table 4) indicated that after 24 hours of culture, 96% of the lead was associated with the total membrane fraction, and about 0.1% was found in the spent media. However, after 96 hours of culture, 98 and 0.3% of the lead were later found in the culture media and membrane fractions, respectively. For both 24 and 96 hours of harvest, only 0.3% of the lead was associated with the cytoplasmic fraction.

Chromium. Table 5 shows the results of the distribution of chromium in the chromium-adapted bacteria. Unlike the lead-adapted bacteria (Table 4), the majority (68%) of the reduced (trivalent) chromium was mobilized in the cytosol. Thirty and 0.3% of the chromium were found in the membrane and in the spent media, respectively.

TABLE 4. DISTRIBUTION OF LEAD IN BACTERIAL SUBFRACTIONS

BACTERIAL FRACTIONS	TOTAL LEAD RECOVERED			
	After 24 hrs.		After 96hrs.	
	Mg	Percent	Mg	Percent
Whole cell wash	0.01±0.00	0.10	0.05±0.01	0.05
Membranes	9.60±1.80	96.00	0.03±0.01	0.30
Cytosol	0.03±0.10	0.30	0.01±0.10	0.10
Culture Media	0.01±2.90	0.10	9.80±3.20	98.00
Control Media	9.90±1.70	99.00	9.80±1.50	98.00

Values are means and standard deviations of nine separate experiments. One-hundred (100) ppm or 10 mg of lead solution was made with Lauryl broth. Each culture was inoculated with 5 milliliters of Lead-adapted bacterial culture. Cultures were harvested after 24 and 96 hours. Bacterial fractions were obtained by differential centrifugation, acid hydrolyzed and assayed for lead with the atomic absorption unit. Control culture was not inoculated with bacteria.

TABLE 5. DISTRIBUTION OF TRIVALENT CHROMIUM IN BACTERIAL SUBFRACTIONS

BACTERIA FRACTIONS	TOTAL % Cr After 24 Hrs.		TOTAL % Cr After 96Hrs.	
	Cr^{+3}	Cr^{+6}	Cr^{+3}	Cr^{+6}
Whole Cell Wash	1.0±0.3	0.3±0.1	5.0±1.8	0.0±0.0
Membranes	30.0±3.2	0.0±0.0	0.5±0.1	0.0±0.0
Cytosol	68.0±2.5	0.0±0.0	0.1±0.1	0.0±0.0
Culture Media	0.3±0.1	0.0±0.0	90.0±5.3	0.1±0.1
Control Media	0.0±0.0	99.6±3.6	0.5±1.8	96.7±4.9

Values are means and standard deviations of nine separate experiments. One hundred (100) ppm or 10 mg of chromium was made with a Lauryl broth. Each culture was inoculated with 5 milliliters of Cr-adapted bacteria per 100 milliliters of medium. Bacterial cells were harvested 24 and 96 hours after culture. The percents of trivalent chromium were determined from differences between total and hexavalent chromium values. Control samples were not inoculated with bacteria.

Similar to the lead culture, after 96 hours, 90% of the trivalent chromium was secreted to the culture medium. Virtually none of the chromium ions were detected in the membrane and cytosolic fractions after 96 hours of culturing. The data (Table 5) also indicated that within 24 hours of culture, 99.7% of the hexavalent chromium had been transformed to the trivalent states. Control samples, without chromium-adapted bacteria indicated no reduction of the hexavalent chromium.

Selenium. Similarly to the lead distribution experiments, 94% of the selenium was detected in the membrane fraction after 24 hours of harvest (Table 6). The absence of selenium in the cytosol at 24 and 96 hours was indicated and significant.

Microscopic examination of cells cultured in the selenium system and gross observations of bacterial colonies on selenium-medium agar plates, clearly indicated a reduction of the selenium (as selenite) to a pink color of elemental selenium. The absence of selenium in the membrane, and cytosolic fractions, after 96 hours of culture, was consistent with the pond observation of selenium increases in the soil and water column of the simulated pond.

TABLE 6. DISTRIBUTION OF SELENIUM IN BACTERIAL SUBFRACTIONS

BACTERIAL FRACTIONS	TOTAL SELENIUM RECOVERED After 24 Hrs.		After 96 Hrs.	
	Mg	Percent	Mg	Percent
Whole Cell Wash	0.1±0.1	2.0	0.5±1.6	10.0
Membranes	3.2±2.3	94.0	0.1±0.3	0.2
Cytosol	0.1±0.1	1.0	0.0±0.0	0.0
Culture Medium	0.1±0.1	0.1	4.3±2.2	86.0
Control Medium	4.9±1.6	99.0	4.9±1.8	99.0

Values are means and standard deviations of nine separate experiments. Each culture containing 50 ppm of selenium was inoculated with 5 ml of Se-adapted bacteria. Cultures were harvested 24 and 96 hours. Bacterial fractions were obtained by differential centrifugation. Each fraction was assayed for selenium using the atomic absorption unit. Control samples were not inoculated with bacteria.

Examination of Bacterial Membrane and Cytosolic Fractions for Metal Mobilization

In the examination of the bacterial fractions for the distribution of the metals (Tables 4, 5, and 6), it was observed that the major sites for the mobilization of lead and selenium were at the membrane site. In the case of chromium, the reduction was primarily at the cytosol.

To correlate the above metal mobilization processes to specific proteins, native PAGE analyses of membranes and cytosol were performed. Figure 16 shows the results of membrane fractions from selenium bacterial cultures. Lane a represented a sample from the wild-type bacteria. Lanes b and c represented the membrane fractions from selenium-adapted bacteria, in the absence and presence of selenium, respectively.

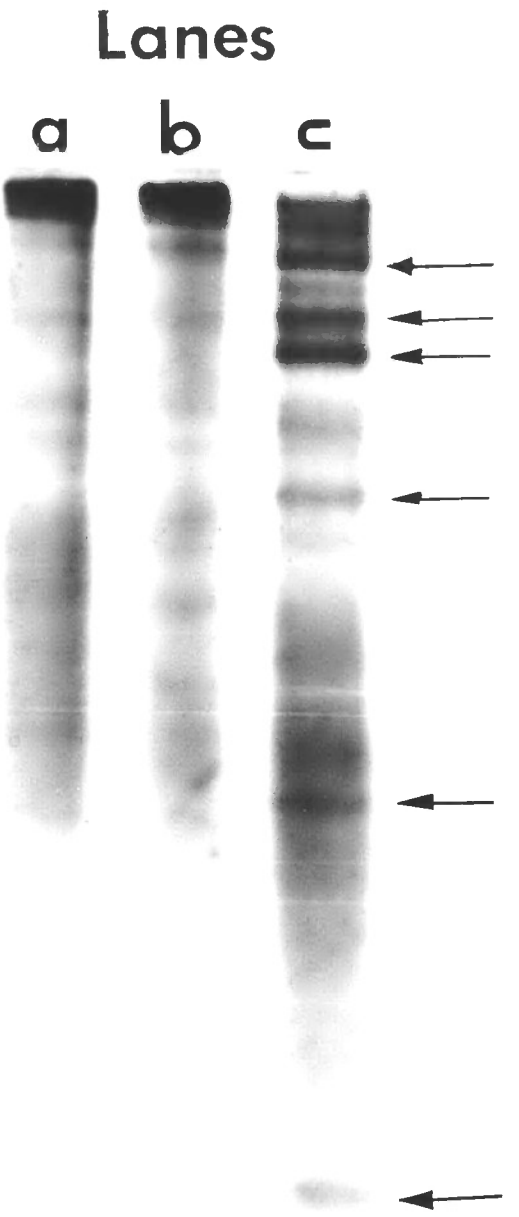
Under the stress of selenium (lane c), one observed specific protein profiles (Shown by the arrows), which when analyzed, indicated the presence of selenium (data not shown). Of significance was the low molecular weight protein, which was previously observed as an extracellular protein (Fig. 15, lane b). The gross observation from figure 16, indicated that, under the stress of selenium, specific proteins were exhibited, which differed in the amount expressed and their mobilities. This observation was consistent with the emergence of resistant bacteria during selenium uptake.

Fig. 16. Native PAGE analyses of membrane fractions of selenium bacterial lysates.

(Lane a) Membrane fraction of wild-type bacteria, in the absence of selenium stress.

(Lane b) Membrane fraction of selenium-adapted baceria, in the absence of selenium stress.

(Lane c) Mebrane fraction of selenium-adapted bacteria under the stress of selenium.


Lanes
a
b
c

Due to the high chromium-reductase activity obtained in the cytosolic fraction from the Cr-adapted bacteria, the cytosolic fraction was analyzed for specific proteins. Figure 17 showed the results of a native PAGE analyses of the cytosolic fraction. Lanes a and b represented cytosolic fractions of Cr-adapted bacteria, with and without the stress of chromium, respectively. Lane c represented the wild-type fraction.

In common, are the low molecular weight proteins observed in all the lanes (a, b, and c). In contrast, however, are the specific proteins in lane a, that were generated when Cr-adapted bacteria were under the stress of chromium. The latter proteins exhibited different mobility patterns compared to lanes b and c. Examination of these specific proteins on lane a, for the presence of chromium, showed the presence of chromium; highest percentage obtained at the high molecular weight protein (data not shown). Also, of significance was lane b, which exhibited two prominent, specific proteins, apparently generated after the adaptation process.

Identification of a Chromium Reductase

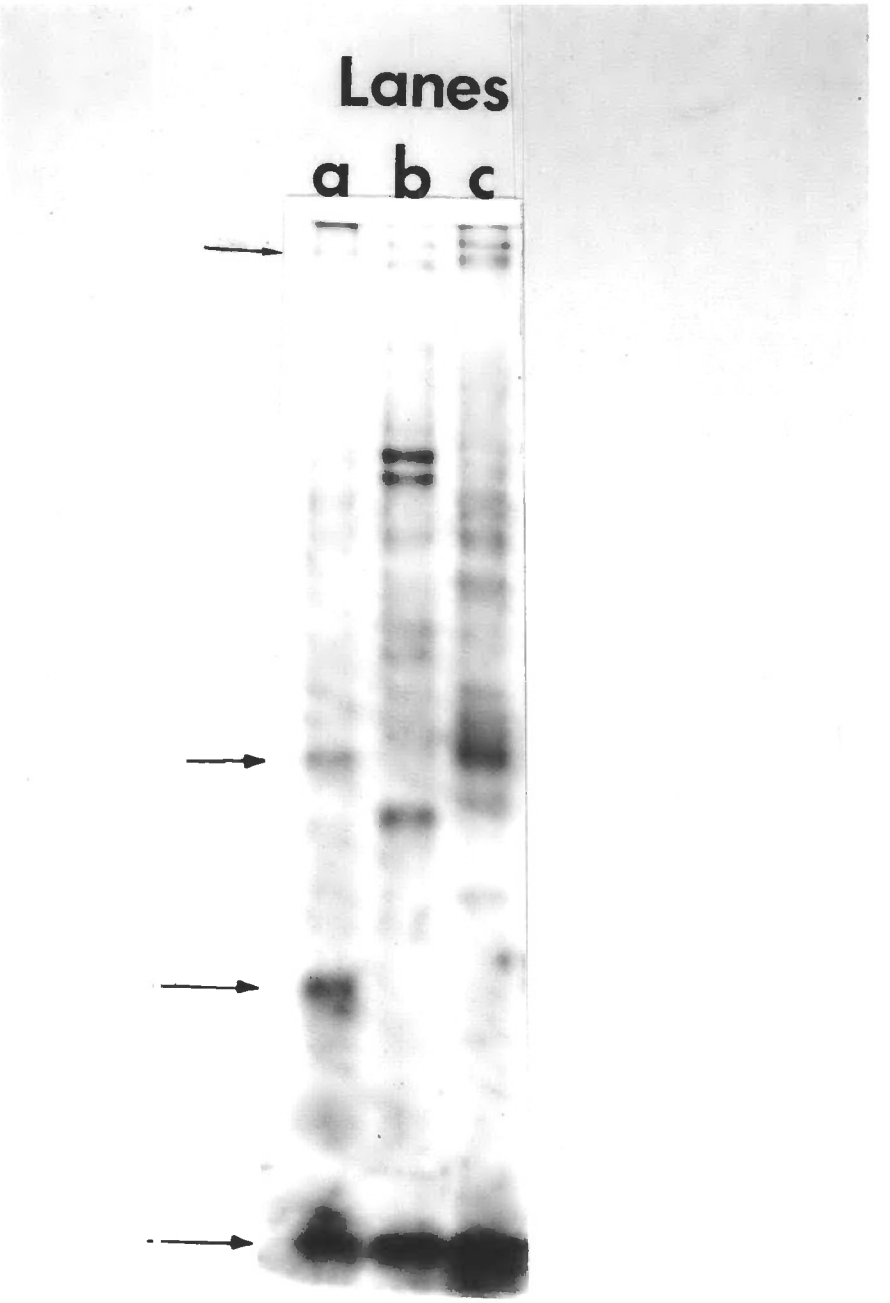
From Table 5, it was noted that after 24 hours of culture, 99% of the hexavalent chromium was reduced to

Fig. 17. Native PAGE analyses of cytosolic fractions of chromium bacterial strains.

(Lane a) Cytosolic fraction of Cr-adapted bacteria under the stress of chromium.

(Lane b) Cytosolic fraction of Cr-adapted bacteria without the stress of chromium.

(Lane c) Cytosolic fraction of wild-type bacteria, grown in the absence of chromium.


Lanes
a b c

the trivalent state. In order to identify the specific protein(s), which may be involved in the chromium reduction process, the total lysate from the chromium-adapted bacteria was analyzed with ion exchange chromatography. Figure 18 (panel A) represents protein readings from 3 milliliter fractions, collected from the ion exchange column. The resulting protein profiles indicated specific protein peaks. Pooled fractions from the ion exchange column were assayed with a spectrophotometer, at 418 nm wavelength reading, in an attempt to correlate fractions to chromium-reductase activity. The 418 nm reading was used after a wavelength scan that identified bound trivalent chromium at a maximum peak of 418 nm. Figure 18 (panel B) indicated that there were strong correlations between the specific protein peaks (Fig. 18, panel A) and the chromium-reductase activity (Fig. 18, panel B).

Analyses of the ion exchange fractions, with atomic absorption, in order to correlate samples with the presence of trivalent chromium, indicated that the major peaks observed in panels A and B corresponded to the presence of trivalent chromium (Fig. 18, panel C).

Native PAGE analyses of pooled fractions (corresponding to the major peaks in figure 18, panel B), revealed specific high molecular weight proteins, that showed similar mobilities (Fig. 19). Figure 19, lanes a,

Fig. 18. Ion exchange chromatography of total lysate of chromium-adapted bacteria.

(Panel A) Protein profiles.

(Panel B) Correlation of protein profiles to chromium-reductase activity.

(Panel C) Correlation of ion exchange fractions to trivalent chromium.

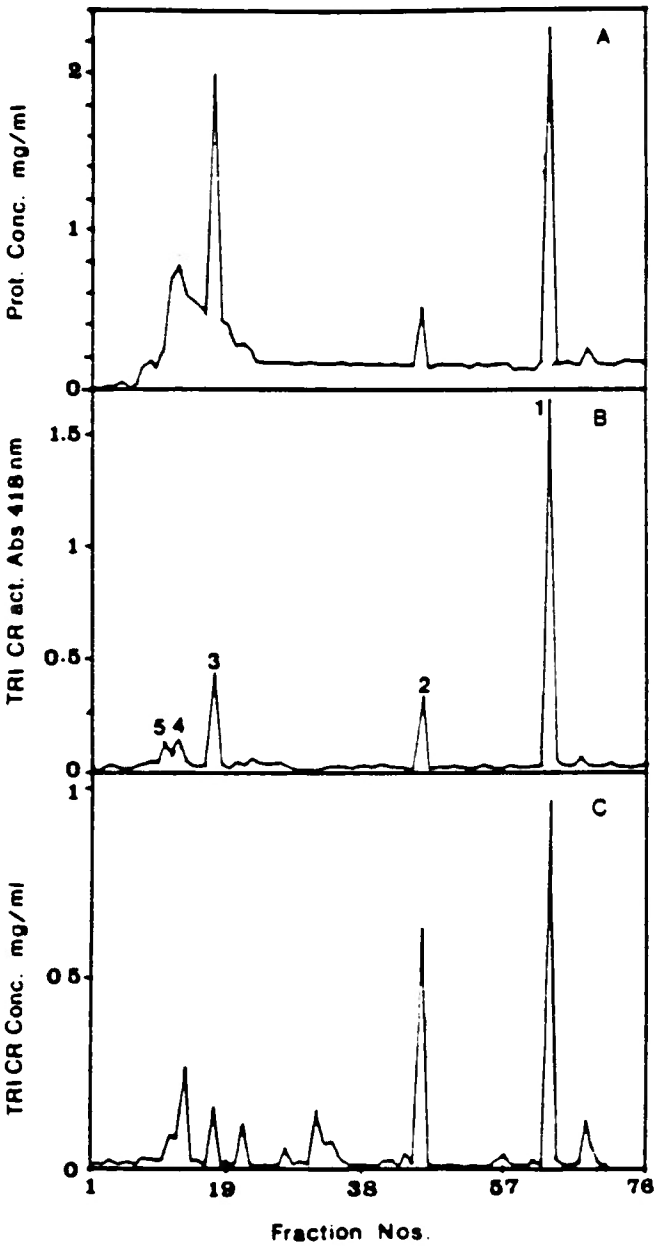

A
Prot. Conc. mg/ml
2
1
0
B
TRI CR act. Abs 418 nm
1.5
1
0.5
0
1
2
3
4
5
C
TRI CR Conc. mg/ml
1
0.5
0
1
19
38
57
76
Fraction Nos.

Fig. 19. Native PAGE analyses of pooled ion exchange fractions.

Protein profiles represent fractions corresponding to chromium-reductase activity (Fig. 18, panel B).

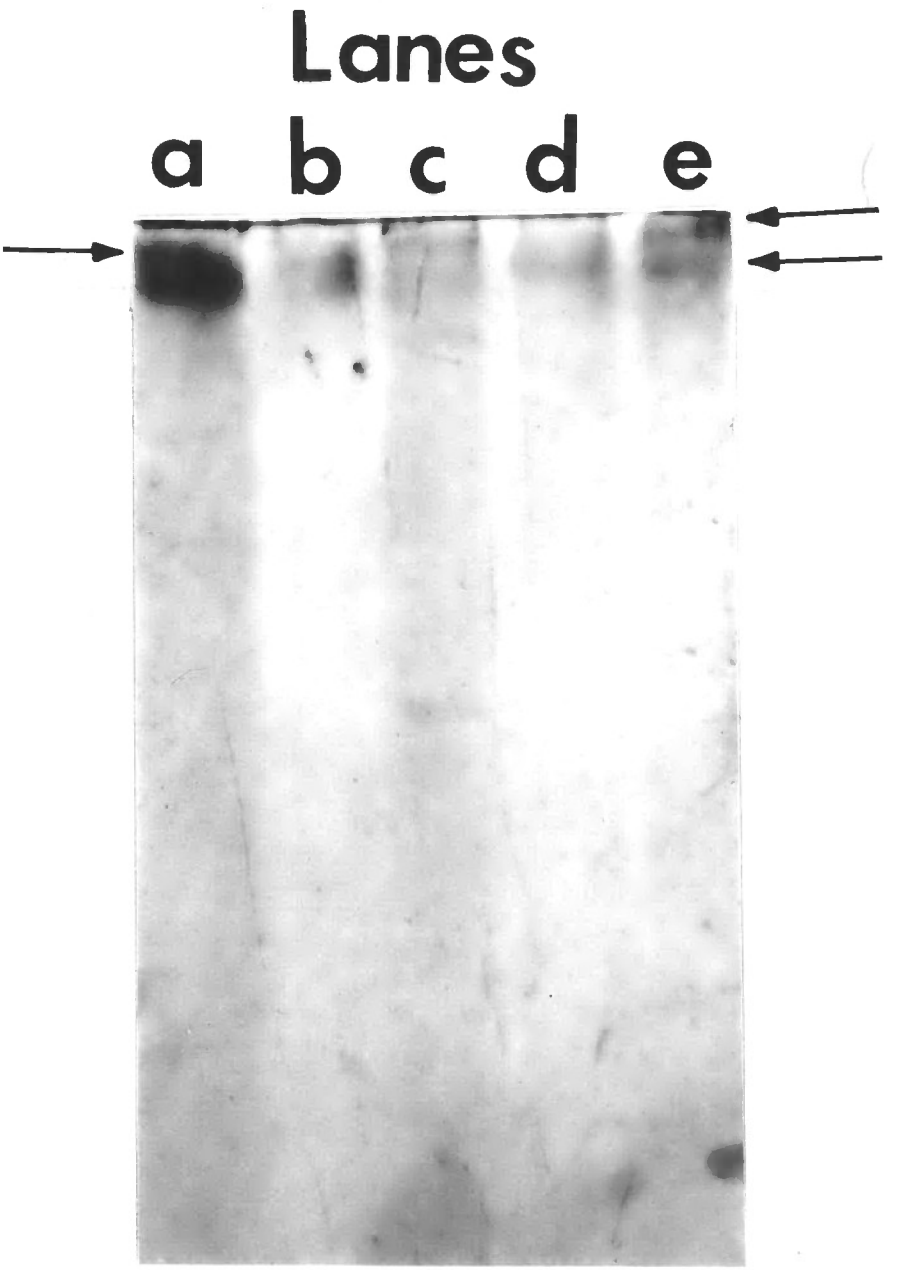
(Lane a) Fraction corresponding to peak 1.

(Lane b) Fraction corresponding to peak 2.

(Lane c) Fraction corresponding to peak 3.

(Lane d) Fraction corresponding to peak 4.

(Lane e) Fraction corresponding to peak 5.


Lanes
a b c d e

b, c, d, and e represented fractions shown in figure 18, panel B, peaks 1, 2, 3, 4, and 5, respectively. From figure 19, lane a, it was clear that the fraction from peak 1, (Fig. 18) has the highest activity, due to the increased expression of the high molecular weight protein isolated. Figure 19, lane e, seems to show two high molecular weight proteins, which probably were a mixture of peaks 4 and 5 of figure 18, panel B. When the proteins from the native PAGE analyses (Fig. 19) were fractioned separately, and assayed for the presence of trivalent chromium, with the atomic absorption unit, lane a (Fig. 19) indicated the greatest (25-fold) amount of trivalent chromium (Table 7). As a result of the above data (Fig. 19 and Table 7), the high molecular weight protein from the native PAGE analyses (Fig. 19, lane a) was fractioned and analyzed on SDS-PAGE, in conjunction with the fraction obtained from figure 19, panel B, peak 1.

Figure 20 shows the results of the SDS-PAGE analyses. Lanes a and b represented the samples from the native gel and the fraction from the ion exchange column, respectively as described above. Lane c represented the standard molecular weight marker. As can be seen, lanes a and b showed proteins that have similar molecular weights. The relative molecular weights were 135,000, 99.000, and 98,000.

Table 7. QUANTITATION OF TRIVALENT CHROMIUM IN PARTIALLY PURIFIED PROTEINS DERIVED FROM A TOTAL LYSATE OF Cr-ADAPTED BACTERIA

Protein lanes.	Cr Conc. (mg/l).
A	25
B	1
C	1
D	1
E	2

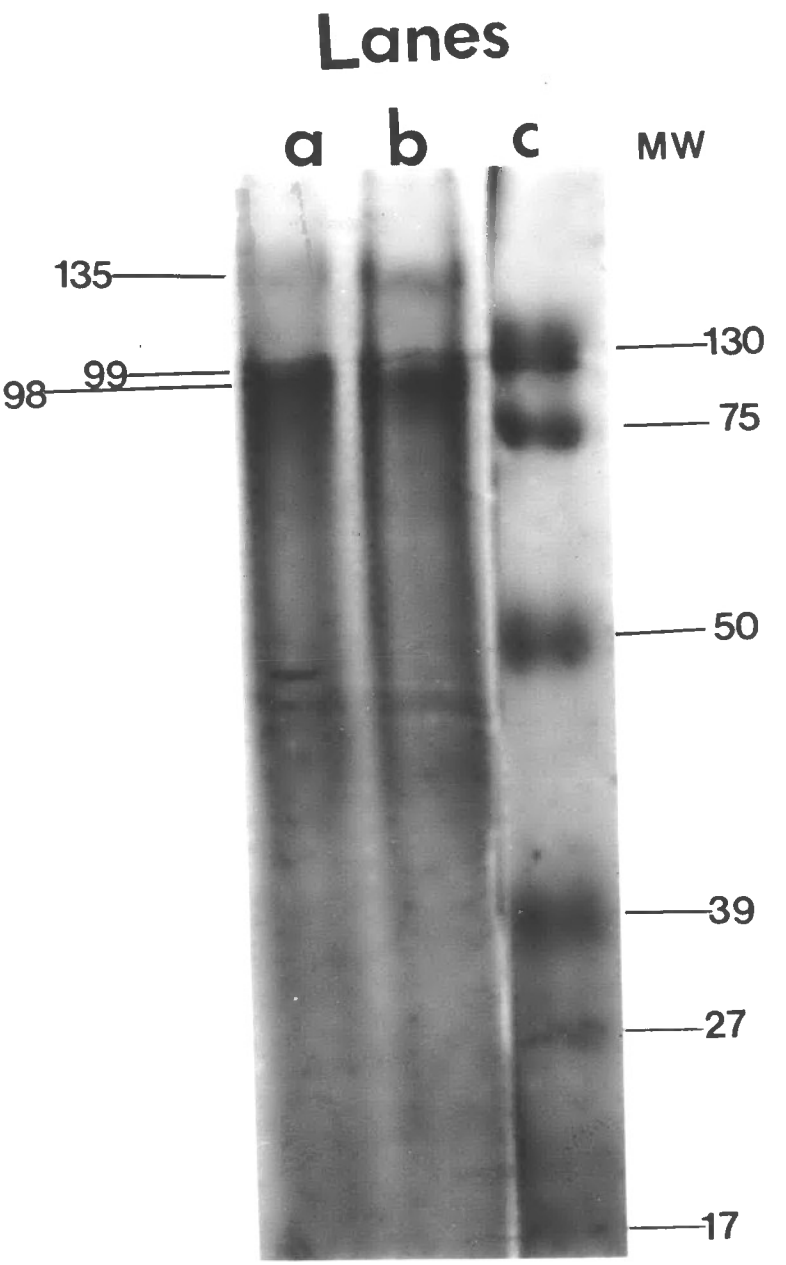
The protein lanes described above correspond to the native PAGE analyses of proteins obtained from ion exchange assay (Fig. 19). Protein bands (Fig. 19, lanes a-e) were fractioned and assayed for trivalent chromium.

Fig. 20. SDS-PAGE analyses of a major chromium-reductase.

(Lane a) Solubilized sample obtained from the native PAGE analyses (Fig. 19, lane a).

(Lane b) Ion exchange sample corresponding to peak 1 of panel B, figure 18.

(Lane c) Standard molecular weight marker.


Lanes
a
b
c
MW
135
99
98
130
75
50
39
27
17

CHAPTER V

DISCUSSION

This investigation has demonstrated the effectiveness of the mixed microbial system in the removal of metals (Pb, Cr, and Se) from contaminated water. In addition, the potential for metal recovery is clearly indicated. The metals which were trapped within the mixed microbial biomass, can be easily harvested by a non-energy intensive mechanism. The study also demonstrated an alternative biotechnology for water and wastewater purification, in which heavy metal recycling was one clear potential (Anon, 1981). A major problem in this system might be the maintenance of the microbes in such heavily metal-infused environments. Heavy metals are known to be very toxic and may also cause fluctuating pH conditions (Strandberg et al., 1979), and the problems inherent in utilizing microbial strains from this source must be considered (Oswald, 1980). An approach to this problem might be the utilization of resistant and adapted microbial strains that may readily absorb the metals before toxic levels are reached in the system. In addition, the detoxification of the metals can be enhanced by the establishment of a functional, reducing chemical environment.

The above premise formed the basis of the development of metal-tolerant microbial strains, which were used in

this study. The choice of the best wild-type organism for a particular assay remains as the important first step for the subsequent improvement of any applied microbiological study (Demain and Solomon, 1986). The process, in general, usually involved repeated applications of three basic processes: (i) induction of genetic variability in cell populations, (ii) assays in small scale of many individuals from those population and (iii) assays with the strains in a larger scale, to allow identification of an improved strain. Each improved strain can then be used as a parent strain in a new cycle of mutation, induction and assay. The prototype system for this adaptation process, probably originated with the work of Thom and Steinberg (1939). The success of these earlier studies has been combined with classical genetic and recombinant DNA programs. The latter studies employed mutant strains, characterized by stable, well defined phenotypic and genotypic properties, to construct special improved strains, of a more defined character. To date, the application of DNA technology has focused on the development of a single genome. In natural systems, the use of pure cultures or isolates has proven to give erroneous conclusions (Barkays, 1987).

The mixed microbial adaptation development ultra-assay (MADU) described in this study demonstrated its uniqueness in the adaptation of metal-tolerant microbes in natural systems. By this technique, the entire microbial population was stressed with increasing

concentrations of the metals. On the final analyses, a mixed metal-tolerant microbial population was selected. It is projected that in biotechnical applications, the most economical methods may be those which employ the natural ecosystems or most closely imitate the natural processes (Oswald, 1980). The adaptation technique described here, evolved a consortium of tolerant microbial species, functioning as an elaborate, cooperative unit.

The above premise was supported by the results represented in (Figs. 5, 8, and 11). The uptake dynamics of the investigated metals (Pb, Cr, and Se) in the presence of the metal-adapted strains were consistent with biological water treatment processes, which employ activated sludge or trickling filters (Oliver and Cosgrove, 1974). However, the pattern of interaction between the microbes and the metals varied with the metal used. For example, the uptake of Pb and Cr was 83% compared to 36% of Se. One possible explanation could be as a result of the deposition of selenium after reduction, as elemental selenium, thus making it unavailable for uptake during mat formation. This study and that of Jonathan et al. (1987) have demonstrated circumstantial evidence which implied the reduction of selenite to elemental selenium. The higher recoveries of lead and chromium may be attributed to the existing chemical environment in the pond which supported the speciation of these metals as $Pb_2Cl(OH)_2$ and $Cr(OH)_3$,

respectively. In these forms, Pb and Cr can easily be bound to the microbial mat, thus increasing their uptake and recovery potentials.

It was obvious from the above discussion, that metal speciation was an important factor in accounting for the uptake efficiency of the metals investigated. It also played a vital role in the understanding of the mechanisms of uptake of these metals in the system. This study demonstrated that strategies, such as chelation, adsorption/absorption, precipitatation, and reduction processes formed part of the complex ways employed by bacteria in the response of Pb, Cr, and Se.

Results from Tables 4, 5, and 6 suggested that about 99% of the Pb and Se was essentially adsorbed and present at the membrane fractions. In differentiating between essentiality versus toxicity, bacterial strains have evolved efflux mechanisms, which allow the nonselective uptake of some toxic metals (Wood and Wang, 1983). Arsenate and cadmium are known to be accidentally taken up by normal transport systems designed for phosphate and zinc (Kaback, 1986). However, resistance to toxicity by these organisms resides in their ability to remove these elements selectively by energy-driven efflux systems (Albert, 1973). Tornabene (1972) has earlier demonstrated the ability of certain Azotobacter sp in the immobilization of Pb ions at their membrane sites. The results obtained in this present study were consistent with these earlier observations.

In Table 5, in contrast to the distribution of lead and selenium in the bacterial subfraction, a greater percentage of the reduced trivalent chromium, was found present in the cytosolic fraction (Table 5). The assumption at this stage, was that this was the consequence of the possible presence of a cytosolic Cr-reductase.

In identifying a Cr-reductase, this study based its finding on the ability of the Cr-adapted bacteria to convert the hexavalent Cr to a lesser toxic form of trivalent Cr. That is, rather than assaying for a specific enzymatic activity, which probably may be true for the description of a pure bacterial isolate, a total enzyme activity was described. In using the latter strategy, it was believed that the total conversion of hexavalent chromium to the trivalent state will provide a more difinitive assay which closely describes the total activity of a consortium of a mixed microbial population in a natural ecosystem. This latter view was supported by the results obtained in the description of mercury (II) reductase (Barkay, 1987). Previous attempts used in the description of the enzyme [mercury (II) reductase] by pure isolates, carrying specific enzymatic activity, were found to be erroneous when challenged in natural systems (Barkay, 1987).

One unique nature of the mixed microbial system described in this study, was the prevalence of a functional chemical environment, which aided tremendously

in the detoxification and complexation of the metal ions. Results presented in Table 1 described the existence of anaerobic zones, which were chemically active in the precipitation of lead as PbS. Although, no direct assay was performed to test for the presence of selenium sulfide, the chemistry of selenium suggests that selenium can form insoluble polysulfides (Rosenfeld, 1984). The presence of both anaerobic and sulfur-reducers within the mat created an ideal chemical environment for the precipitation of selenium as selenium sulfide (Fig. 12).

In addition to the above observations, there was a new and unusual report (Bino et al., 1988) which suggested the possible existence of a sulfur-centered $[Cr(III)]_4$ complex. The significance of this new information was that Cr, at its ground state of trivalent chromium can be coupled and complexed at sulfide existing regions. The phenomenon of metal precipitation at the microbial mat, due to the presence of reducing conditions, was a rather unique aspect of the mixed microbial system used in this study. In addition, the identification of specific proteins in the membrane and cytosolic fractions of the metal-adapted bacterial strains has important implications for metal mobilization and transport and no doubt, contributed to our understanding of some of the mechanisms of metal uptake in the mixed microbial system described in this study.

A significant process has been demonstrated in this study which utilized a mixed microbial system for the

recovery of heavy metals. The increased recovery of these metal (Pb, Cr, and Se) contaminants with a mixed microbial system, can effectively be utilized to mitigate the harzard of toxic and heavy metal contamination of the environment. The latter process (mixed microbial system) also demonstrated a significant potential for the recycling of metal values, which compared favorably with traditional methods of water treatment processes.

CHAPTER V1

SUMMARY AND CONCLUSIONS

1. This investigation has demonstrated the effectiveness of mixed microbial systems in the recovery of heavy metals (Pb, Cr, and Se) from contaminated water.
2. Adaptation of complete ecosystems represents a new approach to the development of metal-tolerant microbes, which possess an increased potential for the detoxification and removal of heavy metals from polluted water.
3. Ion exchange chromatography and SDS-PAGE analyses of lysate obtained from a Cr-adapted bacterial culture, suggested that a major trimeric protein was involved in the reduction of hexavalent chromium to their trivalent states. The relative molecular weights of these proteins are 135,000, 99,000 and 98,000.
4. Atomic absorption analyses of bacterial subfractions suggested that about 99% of the lead and selenium were found associated with the membranes and cell wall fractions.
5. Major mechanisms of microbial response to heavy metals (Pb, Cr, and Se) identified included: (1) reduction by microbial sulfate reactions, (2) reduction by changes in oxidation states, (3) precipitation (4) adsorption and secretion, and (5) chelation by specific proteins.

6. Operationally, the microbial systems would be very much less demanding in terms of complex engineered systems and consumption of chemicals. These factors suggest that this system would be significantly less costly with respect to both capital and operating costs.
7. From a practical standpoint, the data obtained from this investigation are significant in metal treatment processes, and compare favorably with conventional methods of metal precipitation. However, in situations where metal recycling are not practiced, the metal solids, deposited in the biomass may constitute a harzadous waste and must be dealt with as such.

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